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Smith et al.

(54) INTEGRAL MEMBRANE PROTEIN DISPLAY ON POXVIRUS EXTRACELLULAR ENVELOPED VIRIONS

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- (63) Continuation of application No. 16/384,087, filed on Apr. 15, 2019, now Pat. No. 10,550,199, which is a continuation of application No. 16/091,077, filed as application No. PCT/US2017/028787 on Apr. 21, 2017, now Pat. No. 10,577,427.
- (60) Provisional application No. 62/326,501, filed on Apr. 22, 2016.

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	C07K 16/32	(2006.01)
	A61K 39/00	(2006.01)
	C07K 14/005	(2006.01)
	C07K 14/705	(2006.01)
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	C07K 14/71	(2006.01)
	C07K 14/715	(2006.01)
	C07K 14/72	(2006.01)
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C07K 14/7158 (2013.01); C07K 14/723 (2013.01); C07K 16/081 (2013.01); C07K 16/2866 (2013.01); C07K 16/2869 (2013.01); C07K 16/2896 (2013.01); C12N 15/86 (2013.01); C07K 2319/00 (2013.01); C07K 2319/01 (2013.01); C07K 2319/02 (2013.01); C07K 2319/21 (2013.01); C12N 2710/24121 (2013.01); C12N 2710/24121 (2013.01); C12N 2710/24131 (2013.01); C12N 2710/24131 (2013.01); C12N 2710/24143 (2013.01)

(58) Field of Classification Search

CPC .. C07K 2319/00; C07K 14/705; C07K 16/32; A61P 35/00; C12N 15/86

See application file for complete search history.

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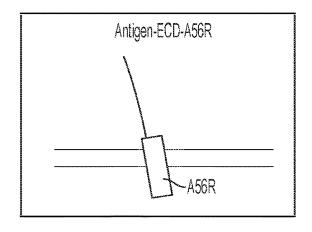
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Primary Examiner — Barry A Chestnut

(57) ABSTRACT

This disclosure provides compositions and methods for expressing and displaying isolated integral membrane proteins (IMPs) or fragments thereof in a native conformation for use in the screening, selecting, and identifying of antibodies or antibody-like molecules that bind to a target IMP of interest.

15 Claims, 11 Drawing Sheets Specification includes a Sequence Listing.



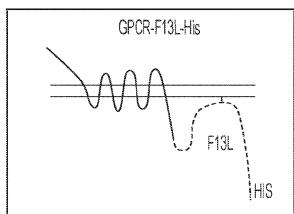


FIG. 1A

FIG. 1B

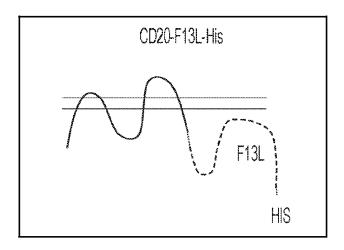


FIG. 1C

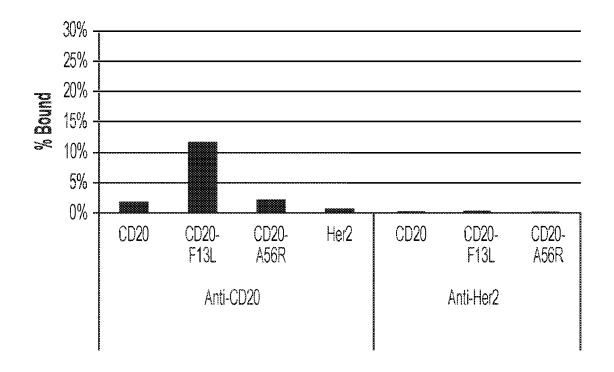
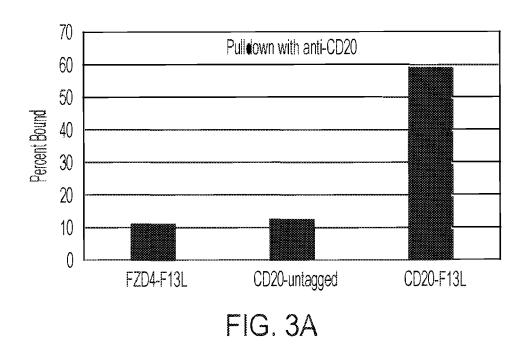


FIG. 2



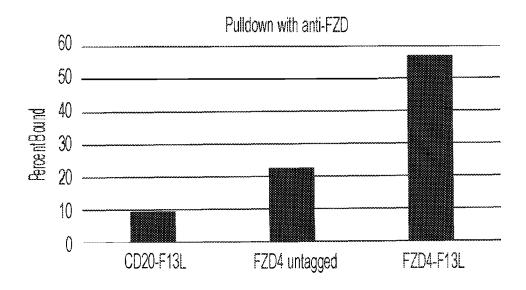
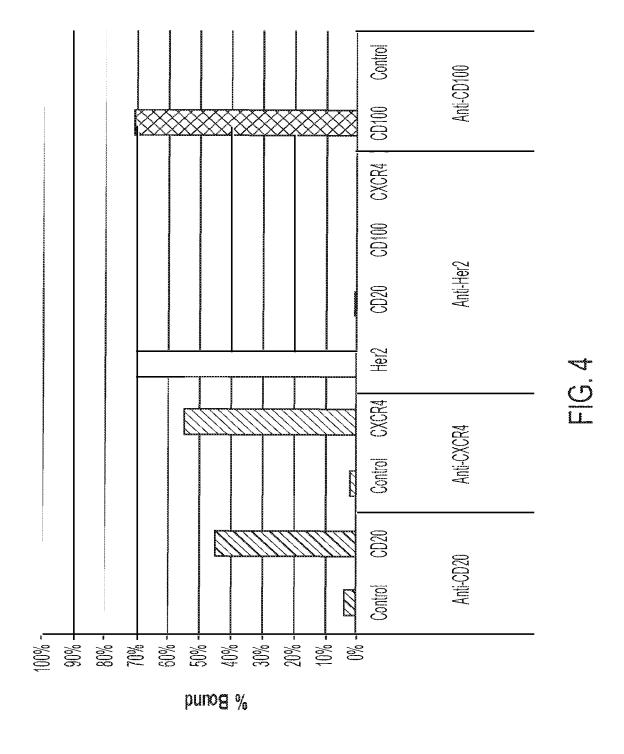
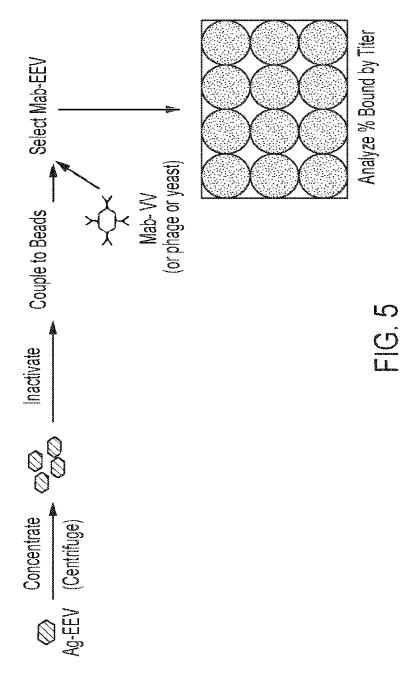
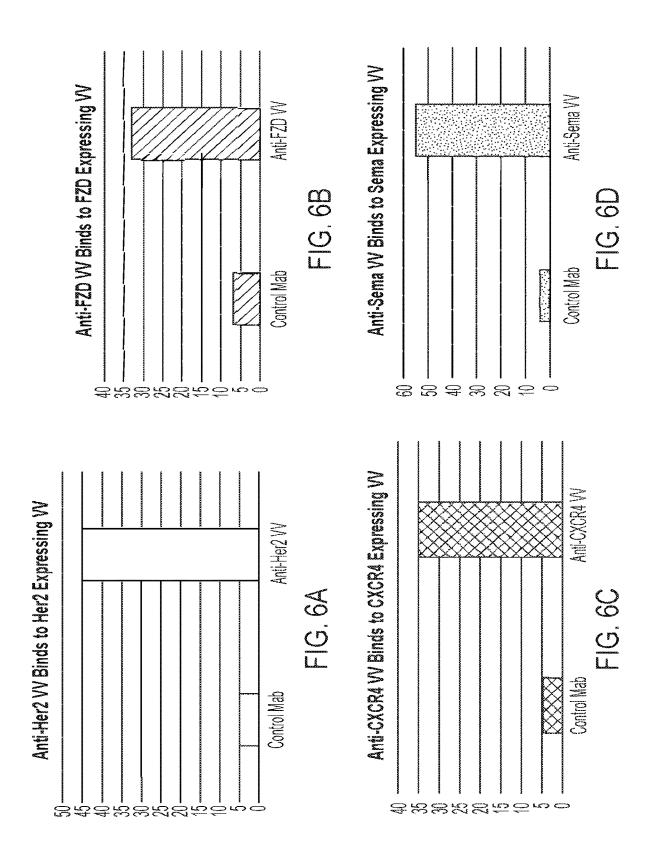
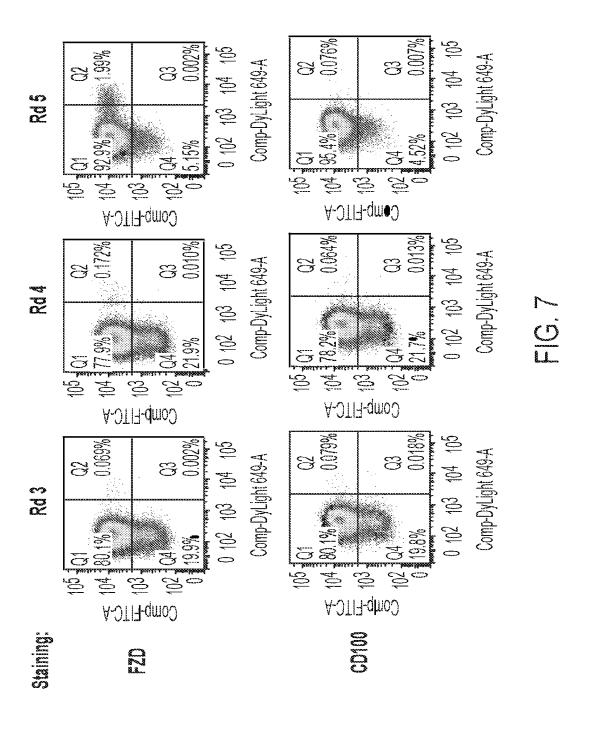


FIG. 3B









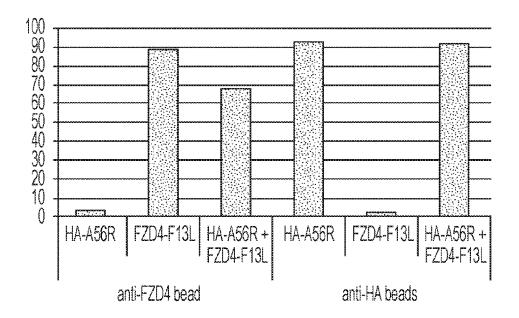


FIG. 8

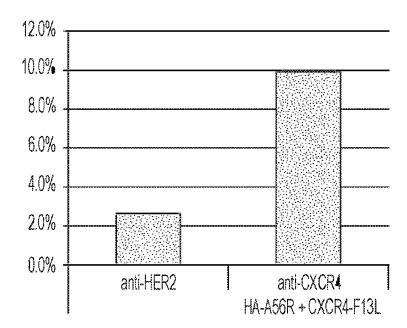


FIG. 9

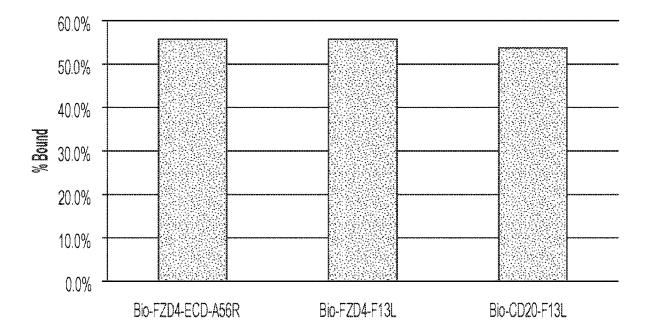


FIG. 10

INTEGRAL MEMBRANE PROTEIN DISPLAY ON POXVIRUS EXTRACELLULAR ENVELOPED VIRIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 16/384,087, filed Apr. 15, 2019, which is a continuation of U.S. patent application Ser. No. 16/091,077, ¹⁰ filed Oct. 3, 2018, which is a U.S. National Stage Entry of PCT Application No. PCT/US2017/028787, filed Apr. 21, 2017, which claims priority benefit of the filing date of U.S. Provisional Patent Application Ser. No. 62/326,501 filed on Apr. 22, 2016, which are each hereby incorporated by ¹⁵ reference in their entireties.

REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY

The content of the electronically submitted sequence listing in ASCII text file (Name "Sequence Listing.txt; Size: 61,440 bytes; and Date of Creation: Oct. 3, 2018") filed with the application is incorporated herein by reference in its entirety.

BACKGROUND

Antibodies of defined specificity are being employed in an increasing number of diverse therapeutic applications. A 30 number of methods have been used to obtain useful antibodies for human therapeutic use. These include chimeric and humanized antibodies, and fully human antibodies selected from libraries, e.g., phage display libraries, or from transgenic animals. Immunoglobulin libraries constructed in 35 bacteriophage can derive from antibody producing cells of naïve or specifically immunized individuals and could, in principle, include new and diverse pairings of human immunoglobulin heavy and light chains. Although this strategy does not suffer from an intrinsic repertoire limitation, it 40 requires that complementarity determining regions (CDRs) of the expressed immunoglobulin fragment be synthesized and fold properly in bacterial cells. Many antigen binding regions, however, are difficult to assemble correctly as a fusion protein in bacterial cells. In addition, the protein will 45 not undergo normal eukaryotic post-translational modifications. As a result, this method imposes a different selective filter on the antibody specificities that can be obtained. Alternatively, fully human antibodies can be isolated from libraries in eukaryotic systems, e.g., yeast display, retroviral 50 display, or expression in DNA viruses such as poxviruses. See, e.g., U.S. Pat. No. 7,858,559, and U.S. Patent Appl. Publication No. 2013-028892, which are incorporated herein by reference in their entireties.

Many important targets for therapeutic antibodies are 55 integral membrane proteins (IMPs), e.g., multi-pass membrane proteins (GPCRs, Ion Channels, etc.) that are difficult to express and purify in a conformationally-intact state. The absence of properly folded target proteins in an isolated state makes the identification and selection of antibodies to these 60 targets challenging. While certain IMPs can be expressed on the surface of cells, e.g., mammalian cells, whole cells are problematic for use in antibody discovery because they are complex antigen mixtures, target expression can be low, and because certain display packages used to construct antibody 65 libraries (e.g., vaccinia virus antibody libraries) can bind to whole cells non-specifically. There remains a need for new

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methods to express and display target IMPs of interest in their native conformation at a sufficient concentration and with minimal competition from other cell proteins to allow for identification and selection of therapeutic antibodies and antibody-like molecules from display libraries.

SUMMARY

This disclosure provides compositions and methods for expressing and displaying isolated integral membrane proteins (IMPs) or fragments thereof in a native conformation for use in the screening, selecting, and identifying of antibodies or antibody-like molecules that bind to a target IMP of interest.

In certain embodiments, the disclosure provides an isolated polynucleotide that includes: a first nucleic acid fragment that encodes an integral membrane protein (IMP) or fragment thereof, where the IMP or fragment thereof includes at least one extra-membrane region, at least one transmembrane domain and at least one intra-membrane region, and where a portion of the first nucleic acid fragment encoding at least one intra-membrane region is situated at the 5' or 3' end of the first nucleic acid fragment; and a second nucleic acid fragment that encodes a vaccinia virus F13L protein or functional fragment thereof, where the second nucleic acid fragment is fused in frame to a portion of the first nucleic acid fragment that encodes an intramembrane region of the IMP. According to these embodiments, a poxvirus infected cell containing the polynucleotide can express an IMP-F13L fusion protein as part of the outer envelope membrane of an extracellular enveloped virion (EEV). In certain aspects, the F13L protein or functional fragment thereof can include the amino acid sequence SEQ ID NO: 1 or a functional fragment thereof. In certain aspects the IMP is a multi-pass membrane protein comprising at least two, at least three, at least four, at least five, at least six or at least seven transmembrane domains. In certain aspects the IMP is a multi-pass membrane protein listed in Table 1.

In certain aspects the multi-pass IMP can have an odd number of transmembrane domains, the 5' end of the first nucleic acid fragment can encode an extra-membrane region, and the 3' end of the first nucleic acid fragment can encode an intra-membrane region fused to the 5' end of the second nucleic acid fragment. In certain aspects the first nucleic acid fragment of this type can encode, e.g., a G-protein coupled receptor (GPCR). In certain aspects the GPCR can be the human frizzled-4 protein (FZD4), or a fragment thereof, and the polynucleotide can encode a polypeptide that includes amino acids 20 to 892 of SEQ ID NO: 2. In certain aspects the polypeptide can further include a signal peptide, e.g., amino acids 1 to 19 of SEQ ID NO: 2. In certain aspects the GPCR can be a CXC chemokine receptor, e.g., CXCR4, or a fragment thereof, and the polynucleotide can encode a polypeptide that includes the amino acid sequence SEQ ID NO: 3.

In certain aspects the multi-pass IMP can have an even number of transmembrane domains, and both the 5' and 3' ends of the first nucleic acid fragment can encode intramembrane regions. In certain aspects, the second nucleic acid fragment can be fused to 3' end of the first nucleic acid fragment. In certain aspects the IMP can be, e.g., human CD20 protein, or a fragment thereof, and the polynucleotide can encode a polypeptide that includes the amino acid sequence SEQ ID NO: 4.

In certain aspects, the first and second nucleic acid fragments of a polynucleotide provided herein can be

directly fused. In certain aspects the polynucleotide as provided herein can include a third nucleic acid fragment encoding a heterologous peptide, e.g., a linker sequence, an amino acid tag or label, or a peptide or polypeptide sequence that facilitates purification, such as a histidine tag. In certain 5 aspects a polynucleotide as provided here can be operably associated with a poxvirus promoter, e.g., a p7.5, a T7, or H5 promoter.

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The disclosure further provides an F13L fusion protein encoded by a polynucleotide as provided herein. The disclosure further provides a poxvirus genome, e.g., a vaccinia virus genome, that includes a polynucleotide as provided herein. The disclosure further provides a recombinant vaccinia virus EEV that includes a poxvirus genome as provided herein.

The disclosure further provides a method of producing a recombinant vaccinia virus EEV as provided herein where the method includes infecting a host cell permissive for vaccinia virus infectivity with a vaccinia virus comprising a poxvirus genome as provided herein, and recovering EEV 20 released from the host cell.

The disclosure further provides a method to display an integral membrane protein (IMP) or fragment thereof in a native conformation where the method includes infecting host cells permissive for poxvirus infectivity with a recom- 25 binant poxvirus that expresses an IMP or fragment thereof as a fusion protein with poxvirus EEV-specific protein or membrane-associated fragment thereof, where EEV produced by the infected host cell comprise the IMP fusion protein as part of the EEV outer envelope membrane and 30 recovering EEV released from the host cell. In certain aspects the IMP or fragment thereof displays on the surface of the EEV in a native conformation. In certain aspects the EEV-specific protein can be the vaccinia virus A33R protein, A34R protein, A56R protein, B5R protein, A36R protein, 35 F13L protein, any membrane-associated fragment thereof, or any combination thereof.

In certain aspects the EEV-specific protein is F13L (SEQ ID NO: 1) or a functional fragment thereof. In certain aspects the IMP is a multi-pass membrane protein that 40 includes at least two, at least three, at least four, at least five, at least six or at least seven transmembrane domains. In certain aspects the IMP can be a G-protein coupled receptor (GPCR), e.g., human FZD4 or CXCR4 as described above, that includes seven transmembrane domains, and the F13L 45 protein can be fused to the C-terminus of the IMP. In certain aspects the IMP or fragment thereof can have an even number of transmembrane domains, e.g., human CD20 as described above, where both the N-terminus and the C-terminus of the IMP or fragment thereof are intra-membrane, 50 and the F13L can be fused to the N-terminus or the C-terminus of the IMP.

In certain aspects the membrane-associated EEV specific protein fragment can include or consist of the stalk, transmembrane, and intra-membrane domains of the vaccinia 55 virus A56R protein, e.g., amino acids 108 to 314 of SEQ ID NO: 5. In certain aspects IMP portion of the A56R fusion protein can include the extracellular domain of human FZD4, e.g., the fusion protein can include amino acids 20 to 370 of SEQ ID NO: 6, the extracellular domain of human ErbB2 (Her2), e.g., the fusion protein can include amino acids 20 to 855 of SEQ ID NO: 7, or the extracellular domain of human CD100 (Semaphorin 4D), e.g., the fusion protein can include amino acids 20 to 935 of SEQ ID NO: 8

In certain aspects the membrane-associated EEV specific protein fragment can include or consist of the transmem-

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brane and intra-membrane domains, or the stalk, transmembrane, and intra-membrane domains of the vaccinia virus B5R protein, e.g., amino acids 276 to 317 of SEQ ID NO: 9 or amino acids 238 to 317 of SEQ ID NO: 9, respectively. In certain aspects the IMP portion of the B5R fusion protein can include the extracellular domain of human FZD4, e.g., the fusion protein can include amino acids 20 to 243 of SEQ ID NO: 10 or amino acids 20 to 281 of SEQ ID NO: 11.

The disclosure further provides a fusion protein comprising: amino acids 20 to 892 of SEQ ID NO: 2; SEQ ID NO: 3; SEQ ID NO: 4; amino acids 20 to 370 of SEQ ID NO: 6; amino acids 20 to 855 of SEQ ID NO: 7; amino acids 20 to 935 of SEQ ID NO: 8; amino acids 20 to 243 of SEQ ID NO: 10; amino acids 20 to 281 of SEQ ID NO: 11, amino acids 20 to 506 of SEQ ID NO: 16, or amino acids 20 to 235 of SEQ ID NO: 17. A fusion protein as provided, when expressed by a recombinant poxvirus, e.g., a vaccinia virus, can appear on the surface of a poxvirus extracellular enveloped virion (EEV) in a native conformation. A recombinant poxvirus EEV comprising the fusion protein is also provided. The disclosure further provides a recombinant poxvirus EEV that includes a heterologous IMP or fragment thereof fused to a poxvirus EEV-specific protein or membrane-associated fragment thereof, where the fusion protein is situated in the EEV outer envelope membrane, and where the IMP or fragment thereof displays on the surface of the EEV in its native conformation. In certain aspects the recombinant poxvirus EEV is a vaccinia virus EEV.

The disclosure further provides a method to select antibodies that bind to a multi-pass membrane protein where the method includes attaching recombinant EEV as provided herein to a solid support; providing an antibody display library, where the library comprises display packages displaying a plurality of antigen binding domains; contacting the display library with the EEV such that display packages displaying antigen binding domains that specifically binds to the IMP expressed on the EEV can bind thereto; removing unbound display packages; and recovering display packages that display an antigen binding domain specific for the IMP expressed on the EEV. In certain aspects of this method the recombinant EEV are inactivated prior to attachment to the solid support, e.g., by incubation with Psoralen (Trioxsalen, 4'-aminomethyl-, hydrochloride) in the presence of UV irradiation. In certain aspects of this method the recombinant EEV are attached to the solid surface via reaction with tosyl groups attached to the surface. In certain aspects the solid surface can be tosyl-activated magnetic beads. In certain aspects of this method the recombinant EEV are biotinylated and attached to a streptavidin coated solid surface, e.g., streptavidin-coated magnetic beads.

BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

FIG. 1A-C: Diagrammatic depiction of integral membrane proteins (IMPs) or fragment thereof fused to vaccinia virus extracellular enveloped virion (EEV)-specific proteins or fragments thereof. The parallel horizontal lines are a diagram of the EEV outer membrane. FIG. 1A diagrams the extracellular domain (ECD) of an IMP fused to a fragment of the vaccinia A56R protein that includes the transmembrane domain and the intra-membrane domain. FIG. 1B diagrams the topology of a typical G protein-coupled receptor fused to the vaccinia virus EEV-specific F13L protein. F13L is associated with the inner side of the EEV outer membrane via palmitoylation. FIG. 1C diagrams the topol-

ogy of an IMP with an even number of transmembrane domains, e.g., CD20), fused to F13L.

FIG. 2: Demonstration of incorporation of CD20-F13L and CD20 ECD-A56R fusion proteins into vaccinia virus EEV particles.

FIG. **3**A: Demonstration of preferential incorporation of CD20-F13L fusion protein over untagged CD20 into vaccinia virus EEV particles.

FIG. **3**B: Demonstration of preferential incorporation of FZD4-F13L fusion protein over untagged (unfused) FZD4 ¹⁰ into vaccinia virus EEV particles.

FIG. 4: Incorporation of additional IMP-EEV protein fusions into vaccinia virus EEV. "CD20" is a CD20-F13L fusion protein, "CXCR4" is a CXCR4-F13L fusion protein, "Her2" is a Her2 ECD-A56R fusion protein; and "CD100" 15 is a CD100 ECD-A56R fusion protein.

FIG. 5: Outline of assay for screening an antibody display library for display packages that bind to an IMP of interest expressed on vaccinia virus EEV.

FIG. **6**A: Binding of vaccinia virus EEV expressing an ²⁰ anti-HER-2 antibody to vaccinia virus EEV expressing the HER2 ECD as a fusion with the vaccinia virus A56R protein, bound by tosyl-groups to magnetic beads.

FIG. **6**B: Binding of vaccinia virus EEV expressing an anti-FZD antibody to vaccinia virus EEV expressing FZD4 ²⁵ as a fusion with the vaccinia virus F13L protein, bound by tosyl-groups to magnetic beads.

FIG. **6**C: Binding of vaccinia virus EEV expressing an anti-CXCR4 antibody to vaccinia virus EEV expressing the CXCR4 as a fusion with the vaccinia virus F13L protein, ³⁰ bound by tosyl-groups to magnetic beads.

FIG. **6**D: Binding of vaccinia virus EEV expressing an anti-CD100 ("sema") antibody to vaccinia virus EEV expressing the CD100 ECD as a fusion with the vaccinia virus A56R protein, bound by tosyl-groups to magnetic ³⁵ beads.

FIG. 7: FACS scans showing enrichment for anti FZD4 antibodies following panning on inactivated FZD-ECD-A45R-expressing EEV bound by tosyl-groups to magnetic beads after 3 (Rd3), 4 (Rd4), and 5 (Rd5) rounds of panning. 40 The top row shows antibody-expressing virus-infected cells stained with 10 μ g/ml FZD-His, followed by anti-His-Dyelight650 and anti-Fab-FITC. The bottom row shows antibody-expressing virus-infected cells stained with 10 μ g/ml CD100-His (negative control), followed by anti-His-Dyelight650 and anti-Fab-FITC.

FIG. 8: Incorporation two different protein fusions (HA-A56R fusion and FZD4-F13L fusion) into vaccinia virus EEV. EEV expressing the HA-A56R fusion alone, the FZD4-F13L fusion alone, or both fusion proteins, were 50 tested for binding to either anti-FZD4-coated beads or anti-HA coated beads.

FIG. 9: Specific recovery of anti-CXCR4-expressing EEV by magnetic beads coated with EEV expressing both an HA-A56R fusion and CXCR4-F13L fusion. The antigen- 55 EEV were coupled to anti-HA coated beads.

FIG. 10: Binding of biotinylated vaccinia virus EEV expressing the designated fusion proteins to streptavidin coated magnetic beads.

DETAILED DESCRIPTION

This disclosure provides methods and compositions for expressing and displaying integral membrane proteins (IMPs), e.g., multi-pass (IMPs), in a conformationally intact 65 or native state on the surface of extracellular enveloped virion particles (EEV) of poxviruses, e.g., vaccinia virus, as

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a fusion with a polypeptide segment an EEV-specific membrane-associated protein, e.g., F13L.

Definitions

The term "a" or "an" entity refers to one or more of that entity; for example, "a binding molecule," is understood to represent one or more binding molecules. As such, the terms "a" (or "an"), "one or more," and "at least one" can be used interchangeably herein.

Furthermore, "and/or" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. Thus, the term and/or" as used in a phrase such as "A and/or B" herein is intended to include "A and B," "A or B," "A" (alone), and "B" (alone). Likewise, the term "and/or" as used in a phrase such as "A, B, and/or C" is intended to encompass each of the following embodiments: A, B, and C; A, B, or C; A or C; A or B; B or C; A and C; A and B; B and C; A (alone); B (alone): and C (alone).

Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure is related. For example, the Concise Dictionary of Biomedicine and Molecular Biology, Juo, Pei-Show, 2nd ed., 2002, CRC Press; The Dictionary of Cell and Molecular Biology, 3rd ed., 1999, Academic Press; and the Oxford Dictionary Of Biochemistry And Molecular Biology, Revised, 2000, Oxford University Press, provide one of skill with a general dictionary of many of the terms used in this disclosure.

Units, prefixes, and symbols are denoted in their Système International de Unites (SI) accepted form. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, amino acid sequences are written left to right in amino to carboxy orientation. The headings provided herein are not limitations of the various aspects or aspects of the disclosure, which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification in its entirety.

As used herein, the term "non-naturally occurring" substance, composition, entity, and/or any combination of substances, compositions, or entities, or any grammatical variants thereof, is a conditional term that explicitly excludes, but only excludes, those forms of the substance, composition, entity, and/or any combination of substances, compositions, or entities that are well-understood by persons of ordinary skill in the art as being "naturally-occurring," or that are, or might be at any time, determined or interpreted by a judge or an administrative or judicial body to be, "naturally-occurring."

As used herein, the term "polypeptide" is intended to encompass a singular "polypeptide" as well as plural "polypeptides," and refers to a molecule composed of monomers (amino acids) linearly linked by amide bonds (also known as peptide bonds). The term "polypeptide" refers to any chain or chains of two or more amino acids, and does not refer to a specific length of the product. Thus, peptides, dipeptides, tripeptides, oligopeptides, "protein," "amino acid chain," or any other term used to refer to a chain or chains of two or more amino acids are included within the definition of "polypeptide," and the term "polypeptide" can be used instead of, or interchangeably with any of these terms. The term "polypeptide" is also intended to refer to the products of post-expression modifications of the polypeptide, including without limitation glycosylation, acetylation, phospho-

rylation, amidation, and derivatization by known protecting/blocking groups, proteolytic cleavage, or modification by non-naturally occurring amino acids. A polypeptide can be derived from a biological source or produced by recombinant technology, but is not necessarily translated from a designated nucleic acid sequence. It can be generated in any manner, including by chemical synthesis.

A polypeptide as disclosed herein can be of a size of about 3 or more, 5 or more, 10 or more, 20 or more, 25 or more, 50 or more, 75 or more, 100 or more, 200 or more, 500 or 10 more, 1,000 or more, or 2,000 or more amino acids. Polypeptides can have a defined three-dimensional structure, although they do not necessarily have such structure. Polypeptides with a defined three-dimensional structure are referred to as folded, and polypeptides that do not possess a 15 defined three-dimensional structure, but rather can adopt a large number of different conformations, and are referred to as unfolded. As used herein, the term glycoprotein refers to a protein coupled to at least one carbohydrate moiety that is attached to the protein via an oxygen-containing or a nitrogen-containing side chain of an amino acid, e.g., a serine or an asparagine.

By an "isolated" polypeptide or a fragment, variant, or derivative thereof is intended a polypeptide that is not in its natural milieu. No particular level of purification is required. 25 For example, an isolated polypeptide can be removed from its native or natural environment. Recombinantly produced polypeptides and proteins expressed in host cells are considered isolated as disclosed herein, as are native or recombinant polypeptides that have been separated, fractionated, 30 or partially or substantially purified by any suitable technique.

As used herein, the term "non-naturally occurring" polypeptide, or any grammatical variants thereof, is a conditional term that explicitly excludes, but only excludes, those forms 35 of the polypeptide that are well-understood by persons of ordinary skill in the art as being "naturally-occurring," or that are, or might be at any time, determined or interpreted by a judge or an administrative or judicial body to be, "naturally-occurring."

Other polypeptides disclosed herein are fragments, derivatives, analogs, or variants of the foregoing polypeptides, and any combination thereof. The terms "fragment," "variant," "derivative" and "analog" as disclosed herein include any polypeptides that retain at least some of the 45 properties of the corresponding native antibody or polypeptide, for example, specifically binding to an antigen. Fragments of polypeptides include, for example, proteolytic fragments, as well as deletion fragments, in addition to specific antibody fragments discussed elsewhere herein. 50 Variants of, e.g., a polypeptide include fragments as described above, and also polypeptides with altered amino acid sequences due to amino acid substitutions, deletions, or insertions. In certain aspects, variants can be non-naturally occurring. Non-naturally occurring variants can be produced 55 using art-known mutagenesis techniques. Variant polypeptides can comprise conservative or non-conservative amino acid substitutions, deletions or additions. Derivatives are polypeptides that have been altered so as to exhibit additional features not found on the original polypeptide. 60 Examples include fusion proteins. Variant polypeptides can also be referred to herein as "polypeptide analogs." As used herein a "derivative" of a polypeptide can also refer to a subject polypeptide having one or more amino acids chemically derivatized by reaction of a functional side group. Also 65 included as "derivatives" are those peptides that contain one or more derivatives of the twenty standard amino acids. For

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example, 4-hydroxyproline can be substituted for proline; 5-hydroxylysine can be substituted for lysine; 3-methylhistidine can be substituted for histidine; homoserine can be substituted for serine; and ornithine can be substituted for lysine.

A "conservative amino acid substitution" is one in which one amino acid is replaced with another amino acid having a similar side chain. Families of amino acids having similar side chains have been defined in the art, including basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). For example, substitution of a phenylalanine for a tyrosine is a conservative substitution. In certain embodiments, conservative substitutions in the sequences of the polypeptides and antibodies of the present disclosure do not abrogate the binding of the polypeptide or antibody containing the amino acid sequence, to the antigen to which the binding molecule binds. Methods of identifying nucleotide and amino acid conservative substitutions that do not eliminate antigen binding are wellknown in the art (see, e.g., Brummell et al., Biochem. 32:1180-1 187 (1993); Kobayashi et al., Protein Eng. 12 (10):879-884 (1999); and Burks et al., Proc. Natl. Acad. Sci. USA 94:412-417 (1997)).

As used herein the term "integral membrane protein" or "IMP" refers to a protein or polypeptide that is attached to a biological membrane. One example of an IMP is a transmembrane protein, which spans the lipid bilayer of the biological membrane one or more times. Single-pass membrane proteins cross the membrane only once, while multipass membrane proteins weave in and out, crossing several times. Type I single-pass proteins are positioned with their amino terminus on the outer side of the membrane or "extra-membrane" and their carboxyl-terminus on the inte-40 rior side of the membrane, or "intra-membrane." Type II single-pass proteins have their amino-terminus on the intramembrane side. Multi-pass transmembrane proteins pass through the membrane two or more times and can have a variety of different topologies. Those proteins with an even number of transmembrane domains will have both their amino terminus and carboxy terminus on the same side of the membrane. One example of such a protein is CD20. which is expressed on B cells. Those with an odd number of transmembrane domains will have their amino- and carboxy termini on opposite sides of the membrane. Examples include G-protein coupled receptors, which typically have 7 transmembrane domains, with the amino terminus on the extra-membrane side and the carboxy terminus on the intramembrane side. Certain IMPs do not have transmembrane domains and are instead anchored to the membrane, e.g., via a lipid such as glycosylphosphatidylinositol or palmitoyl group. IMPs have myriad biological functions including, but not limited to transporters, linkers, channels, receptors, enzymes, energy transduction or cell adhesion.

The term "polynucleotide" is intended to encompass a singular nucleic acid as well as plural nucleic acids, and refers to an isolated nucleic acid molecule or construct, e.g., messenger RNA (mRNA), cDNA, or plasmid DNA (pDNA). A polynucleotide can comprise a conventional phosphodiester bond or a non-conventional bond (e.g., an amide bond, such as found in peptide nucleic acids (PNA)). The terms "nucleic acid" or "nucleic acid sequence" refer to

any one or more nucleic acid segments, e.g., DNA or RNA fragments, present in a polynucleotide.

By an "isolated" nucleic acid or polynucleotide is intended any form of the nucleic acid or polynucleotide that is separated from its native environment. For example, 5 gel-purified polynucleotide, or a recombinant polynucleotide encoding a polypeptide contained in a vector would be considered to be "isolated." Also, a polynucleotide segment, e.g., a PCR product, that has been engineered to have restriction sites for cloning is considered to be "isolated." Further examples of an isolated polynucleotide include recombinant polynucleotides maintained in heterologous host cells or purified (partially or substantially) polynucleotides in a non-native solution such as a buffer or saline. Isolated RNA molecules include in vivo or in vitro RNA 15 transcripts of polynucleotides, where the transcript is not one that would be found in nature. Isolated polynucleotides or nucleic acids further include such molecules produced synthetically. In addition, polynucleotide or a nucleic acid can be or can include a regulatory element such as a 20 promoter, ribosome binding site, or a transcription termina-

As used herein, a "non-naturally occurring" polynucleotide, or any grammatical variants thereof, is a conditional definition that explicitly excludes, but only excludes, those 25 forms of the polynucleotide that are well-understood by persons of ordinary skill in the art as being "naturallyoccurring," or that are, or that might be at any time, determined or interpreted by a judge or an administrative or judicial body to be, "naturally-occurring."

As used herein, a "coding region" is a portion of nucleic acid that consists of codons translated into amino acids. Although a "stop codon" (TAG, TGA, or TAA) is not translated into an amino acid, it can be considered to be part of a coding region, but any flanking sequences, for example 35 promoters, ribosome binding sites, transcriptional terminators, introns, and the like, are not part of a coding region. Two or more coding regions can be present in a single polynucleotide construct, e.g., on a single vector, or in ent) vectors. Furthermore, any vector can contain a single coding region, or can comprise two or more coding regions, e.g., a single vector can separately encode an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region. In addition, a vector, polynucleotide, 45 or nucleic acid can include heterologous coding regions, either fused or unfused to another coding region. Heterologous coding regions include without limitation, those encoding specialized elements or motifs, such as a secretory signal peptide or a heterologous functional domain.

In certain embodiments, the polynucleotide or nucleic acid is DNA. In the case of DNA, a polynucleotide comprising a nucleic acid that encodes a polypeptide normally can include a promoter and/or other transcription or translation control elements operably associated with one or more 55 coding regions. An operable association is when a coding region for a gene product, e.g., a polypeptide, is associated with one or more regulatory sequences in such a way as to place expression of the gene product under the influence or control of the regulatory sequence(s). Two DNA fragments 60 (such as a polypeptide coding region and a promoter associated therewith) are "operably associated" if induction of promoter function results in the transcription of mRNA encoding the desired gene product and if the nature of the linkage between the two DNA fragments does not interfere with the ability of the expression regulatory sequences to direct the expression of the gene product or interfere with the

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ability of the DNA template to be transcribed. Thus, a promoter region would be operably associated with a nucleic acid encoding a polypeptide if the promoter was capable of effecting transcription of that nucleic acid. The promoter can be a cell-specific promoter that directs substantial transcription of the DNA in predetermined cells. Other transcription control elements, besides a promoter, for example enhancers, operators, repressors, and transcription termination signals, can be operably associated with the polynucleotide to direct cell-specific transcription.

A variety of transcription control regions are known to those skilled in the art. These include, without limitation, transcription control regions that function in vertebrate cells, such as, but not limited to, promoter and enhancer segments from cytomegaloviruses (the immediate early promoter, in conjunction with intron-A), simian virus 40 (the early promoter), and retroviruses (such as Rous sarcoma virus). Other transcription control regions include those derived from vertebrate genes such as actin, heat shock protein, bovine growth hormone and rabbit β-globin, as well as other sequences capable of controlling gene expression in eukaryotic cells. Additional suitable transcription control regions include tissue-specific promoters and enhancers as well as lymphokine-inducible promoters (e.g., promoters inducible by interferons or interleukins).

Poxvirus promoters (e.g. p7.5 or H5) or the bacteriophage T7 promoter can also be used as transcription control regions. When employing a T7 promoter, an inducible vaccinia expression system can be utilized. The vaccinia expression system can include, but is not limited, to a first recombinant vaccinia virus that encodes the entire bacteriophage T7 gene 1 coding region for T7 RNA polymerase, and a second recombinant vaccinia virus that encodes a gene of interest flanked by a T7 promoter and termination regulatory elements. Dual infection of eukaryotic cells with both recombinant vaccinia viruses results in synthesis of the T7 RNA polymerase and expression of the gene of interest controlled by the T7 promoter.

Similarly, a variety of translation control elements are separate polynucleotide constructs, e.g., on separate (differ- 40 known to those of ordinary skill in the art. These include, but are not limited to ribosome binding sites, translation initiation and termination codons, and elements derived from picornaviruses (particularly an internal ribosome entry site, or IRES, also referred to as a CITE sequence).

> In other embodiments, a polynucleotide can be RNA, for example, in the form of messenger RNA (mRNA), transfer RNA, or ribosomal RNA.

Polynucleotide and nucleic acid coding regions can be associated with additional coding regions that encode secre-50 tory or signal peptides, which direct the secretion of a polypeptide encoded by a polynucleotide as disclosed herein. According to the signal hypothesis, proteins secreted by mammalian cells have a signal peptide or secretory leader sequence that is cleaved from the mature protein once export of the growing protein chain across the rough endoplasmic reticulum has been initiated. Those of ordinary skill in the art are aware that polypeptides secreted by vertebrate cells can have a signal peptide fused to the N-terminus of the polypeptide, which is cleaved from the complete or "full length" polypeptide to produce a secreted or "mature" form of the polypeptide. In certain embodiments, the native signal peptide, e.g., an immunoglobulin heavy chain or light chain signal peptide is used, or a functional derivative of that sequence that retains the ability to direct the secretion of the polypeptide that is operably associated with it. Alternatively, a heterologous mammalian signal peptide, or a functional derivative thereof, can be used. For example, the wild-type

leader sequence can be substituted with the leader sequence of human tissue plasminogen activator (TPA) or mouse β -glucuronidase.

As used herein, a "library" is a representative genus of polynucleotides, e.g., a group of polynucleotides related 5 through, for example, their origin from a single animal species, tissue type, organ, or cell type, where the library collectively comprises at least two different species within a given genus of polynucleotides. A library of polynucleotides can include, e.g., at least two, at least 5, at least 10, 100, 10³, 10 10^4 , 10^5 , 10^6 , 10^7 , 10^8 , or 10^9 different species within a given genus of polynucleotides. In certain aspects, a library of polynucleotides as provided herein can encode a plurality of polypeptides that contains a polypeptide of interest. In certain aspects, a library of polynucleotides as provided 15 herein can encode a plurality of immunoglobulin subunit polypeptides, e.g., heavy chain subunit polypeptides or light chain subunit polypeptides. In this context, a "library" as provided herein comprises polynucleotides of a common genus, the genus being polynucleotides encoding immuno- 20 globulin subunit polypeptides of a certain type and class e.g., a library might encode a human μ , γ -1, γ -2, γ -3, γ -4, α -1, α -2, $\epsilon,$ or δ heavy chain, or a human κ or λ light chain. Although each member of any one library constructed according to the methods provided herein can encode the same heavy or light 25 chain constant region and/or a membrane anchoring domain, the library can collectively comprise at least two, at least 5, or at least 10, 100, 10³, 10⁴, 10⁵, 10⁶, 10⁷, 10⁸, or 10⁹ different variable region associated with the common constant region.

In other embodiments, the library can a plurality of immunoglobulin single-chain fragments that comprise a variable region, such as a light chain variable region or a heavy chain variable region, and/or both a light chain variable region and a heavy chain variable region, e.g., an 35 ScFv fragment.

As used herein, a "display library" is a library of polynucleotides each carried in a "display package" that expresses the polypeptide encoded by the library polynucleotide on its surface. An antibody display library, for 40 example, can include plurality of display packages, each displaying an antigen binding domain of an antibody on its surface. When the display library is permitted to interact with an antigen of interest, e.g., immobilized on a solid surface, those display packages that bind the antigen can be 45 isolated from the rest of the library and recovered. The polynucleotide encoding the antigen binding domain displayed on the surface of the display package can then be isolated. Display libraries include, without limitation, phage display libraries in bacteria or libraries in eukaryotic sys- 50 tems, e.g., yeast display, retroviral display, or expression in DNA viruses such as poxviruses. See, e.g., U.S. Pat. No. 7,858,559, and U.S. Patent Appl. Publication No. 2013-028892, which are incorporated herein by reference in their entireties. In certain aspects, an antibody display library can 55 be prepared in a poxvirus, e.g., vaccinia virus vector, as fusion proteins with an EEV-specific protein, such that the "display packages" are EEV particles. See U.S. Patent Appl. Publication No. 2013-028892.

Such display libraries can be screened against the IMP 60 fusion proteins displayed on the surface of EEV as provided herein.

By "recipient cell" or "host cell" or "cell" is meant a cell or population of cells in which a recombinant protein can be expressed, a virus can be propagated, or polynucleotide 65 libraries as provided herein can be constructed and/or propagated. A host cell as provided herein is typically a eukaryotic

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cell or cell line, e.g., a vertebrate, mammalian, rodent, mouse, primate, or human cell or cell line. By "a population of host cells" is meant a group of cultured cells which a "library" as provided herein can be constructed, propagated, and/or expressed. Any host cell which is permissive for vaccinia virus infectivity is suitable for the methods provided by this disclosure. Host cells for use in the methods provided herein can be adherent, e.g., host cells that grow attached to a solid substrate, or, alternatively, the host cells can be in suspension.

Host cells as provided herein can comprise a constitutive secretory pathway, where proteins, e.g., proteins of interest expressed by the cell or by a library, are secreted from the interior of the cell either to be expressed on a cell or viral membrane surface or to be fully secreted as soluble polypeptides. In certain aspects, proteins of interest expressed on or in a biological membrane, e.g., an IMP, are expressed on the surface of an enveloped virus produced by the host cell, e.g., an extracellular enveloped vaccinia virus, or EEV. IMPS can follow the same pathway as fully secreted forms or proteins, passing through to the ER lumen, except that they can be retained in the ER membrane by the presence of one or more stop-transfer signals, or "transmembrane domains." Transmembrane domains are hydrophobic stretches of about 20 amino acids that adopt an alpha-helical conformation as they transverse the membrane. Membrane embedded proteins are anchored in the phospholipid bilayer of the plasma membrane. Transmembrane forms of polypeptides of interest, e.g., membrane-anchored immunoglobulin heavy chain polypeptides typically utilize amino terminal signal peptides as do fully secreted forms.

Signal peptides, transmembrane domains, and cytosolic or "intra-membrane" domains are known for a wide variety of membrane bound and/or fully secreted proteins.

Suitable transmembrane domains can include, but are not limited to the TM domain of the vaccinia virus EEV-specific HA protein A56R, or the EEV-specific vaccinia virus transmembrane proteins A33R, A34R, A36R, or B5R. See, e.g., U.S. Patent Appl. Publ. No. 2013/0288927, published Oct. 31, 2013, and incorporated herein by reference in its entirety. In certain aspects the EEV specific protein can be anchored to the inner surface of the viral envelope via a palmitoyl group, e.g., the vaccinia virus protein F13L, discussed in more detail elsewhere herein.

As used herein, the term "binding molecule" refers in its broadest sense to a molecule that specifically binds to a receptor, e.g., an epitope or an antigenic determinant. As described further herein, a binding molecule can comprise one or more "antigen binding domains" described herein. A non-limiting example of a binding molecule is an antibody or fragment thereof that retains antigen-specific binding.

The terms "binding domain" and "antigen binding domain" are used interchangeably herein and refer to a region of a binding molecule that is necessary and sufficient to specifically bind to an epitope. For example, an "Fv," e.g., a variable heavy chain and variable light chain of an antibody, either as two separate polypeptide subunits or as a single chain, is considered to be a "binding domain."

Other antigen binding domains include, without limitation, the variable heavy chain (VHH) of an antibody derived from a camelid species, or six immunoglobulin complementarity determining regions (CDRs) expressed in a fibronectin scaffold.

The terms "antibody" and "immunoglobulin" can be used interchangeably herein. An antibody (or a fragment, variant, or derivative thereof as disclosed herein) includes at least the variable region of a heavy chain (e.g., for camelid species)

or at least the variable regions of a heavy chain and a light chain. Basic immunoglobulin structures in vertebrate systems are relatively well understood. See, e.g., Harlow et al., Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988). Unless otherwise stated, 5 the term "antibody" encompasses anything ranging from a small antigen binding fragment of an antibody to a full sized antibody, e.g., an IgG antibody that includes two complete heavy chains and two complete light chains.

The term "immunoglobulin" comprises various broad 10 classes of polypeptides that can be distinguished biochemically. Those skilled in the art will appreciate that heavy chains are classified as gamma, mu, alpha, delta, or epsilon, $(\gamma, \mu, \alpha, \delta, \epsilon)$ with some subclasses among them (e.g., $\gamma 1$ - $\gamma 4$ or $\alpha 1$ - $\alpha 2$)). It is the nature of this chain that determines the 15 "class" of the antibody as IgG, IgM, IgA IgG, or IgE, respectively. The immunoglobulin subclasses (isotypes) e.g., IgG₁, IgG₂, IgG₃, IgG₄, IgA₁, IgA₂, etc. are well characterized and are known to confer functional specialization.

Light chains are classified as either kappa or lambda (κ , λ). Each heavy chain class can be bound with either a kappa or lambda light chain. In general, the light and heavy chains are covalently bonded to each other, and the "tail" portions of the two heavy chains are bonded to each other by covalent 25 disulfide linkages or non-covalent linkages when the immunoglobulins are generated either by hybridomas, B cells or genetically engineered host cells. In the heavy chain, the amino acid sequences run from an N-terminus at the forked ends of the Y configuration to the C-terminus at the bottom 30 of each chain. The basic structure of certain antibodies, e.g., IgG antibodies, includes two heavy chain subunits and two light chain subunits covalently connected via disulfide bonds to form a "Y" structure, also referred to herein as an "H2L2" structure.

The term "epitope" includes any molecular determinant capable of specific binding to an antibody. In certain aspects, an epitope can include chemically active surface groupings of molecules such as amino acids, sugar side chains, phosphoryl, or sulfonyl, and, in certain aspects, can have three 40 dimensional structural characteristics, and or specific charge characteristics. An epitope is a region of a target that is bound by an antibody.

The term "target" is used in the broadest sense to include substances that can be bound by a binding molecule. A target 45 can be, e.g., a polypeptide, a nucleic acid, a carbohydrate, a lipid, or other molecule. Moreover, a "target" can, for example, be a cell, an organ, or an organism that comprises an epitope bound that can be bound by a binding molecule.

Both the light and heavy chains are divided into regions 50 of structural and functional homology. The terms "constant" and "variable" are used functionally. In this regard, it will be appreciated that the variable regions (which can be called "variable domains" interchangeably herein) of both the variable light (VL) and variable heavy (VH) chain portions 55 determine antigen recognition and specificity. Conversely, the constant domains of the light chain (CL) and the heavy chain (e.g., CH1, CH2 or CH3) confer biological properties such as secretion, transplacental mobility, Fc receptor binding, complement binding, and the like. By convention the 60 numbering of the constant region domains increases as they become more distal from the antigen binding site or aminoterminus of the antibody. The N-terminal portion is a variable region and at the C-terminal portion is a constant region; the CH3 (or CH4 in the case of IgM) and CL 65 domains are at the carboxy-terminus of the heavy and light chain, respectively.

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The six "complementarity determining regions" or "CDRs" present in an antibody antigen binding domain are short, non-contiguous sequences of amino acids that are specifically positioned to form the antigen binding domain as the antibody assumes its three dimensional configuration in an aqueous environment. The remainder of the amino acids in the antigen binding domain, referred to as "framework" regions, show less inter-molecular variability. The framework regions largely adopt a β-sheet conformation and the CDRs form loops that connect, and in some cases form part of, the β-sheet structure. Thus, framework regions act to form a scaffold that provides for positioning the CDRs in correct orientation by inter-chain, non-covalent interactions. The antigen binding domain formed by the positioned CDRs defines a surface complementary to the epitope on the immunoreactive antigen. This complementary surface promotes the non-covalent binding of the antibody to its cognate epitope. The amino acids that make up the CDRs and the framework regions, respectively, can be readily identified for any given heavy or light chain variable region by one of ordinary skill in the art, since they have been defined in various different ways (see, "Sequences of Proteins of Immunological Interest," Kabat, E., et al., U.S. Department of Health and Human Services, (1983); and Chothia and Lesk, J. Mol. Biol., 196:901-917 (1987), which are incorporated herein by reference in their entireties).

In the case where there are two or more definitions of a term that is used and/or accepted within the art, the definition of the term as used herein is intended to include all such meanings unless explicitly stated to the contrary. A specific example is the use of the term "complementarity determining region" ("CDR") to describe the non-contiguous antigen combining sites found within the variable region of both heavy and light chain polypeptides. These particular regions 35 have been described, for example, by Kabat et al., U.S. Dept. of Health and Human Services, "Sequences of Proteins of Immunological Interest" (1983) and by Chothia et al., J. Mol. Biol. 196:901-917 (1987), which are incorporated herein by reference. Immunoglobulin variable domains can also be analyzed, e.g., using the IMGT information system (www://imgt.cines.fr/) (IMGT®/V-Quest) to identify variable region segments, including CDRs. (See, e.g., Brochet et al., Nucl. Acids Res., 36:W503-508, 2008).

Kabat et al. also defined a numbering system for variable domain sequences that is applicable to any antibody. One of ordinary skill in the art can unambiguously assign this system of "Kabat numbering" to any variable domain sequence, without reliance on any experimental data beyond the sequence itself. As used herein, "Kabat numbering" refers to the numbering system set forth by Kabat et al., U.S. Dept. of Health and Human Services, "Sequence of Proteins of Immunological Interest" (1983). Unless use of the Kabat numbering system is explicitly noted, however, consecutive numbering is used for all amino acid sequences in this disclosure.

Binding molecules, e.g., antibodies or antigen binding fragments, variants, or derivatives thereof include, but are not limited to, polyclonal, monoclonal, human, humanized, or chimeric antibodies, single chain antibodies, epitope-binding fragments, e.g., Fab, Fab' and F(ab')₂, Fd, Fvs, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv), single domain antibodies such as camelid VHH antibodies, fragments comprising either a VL or VH domain, fragments produced by a Fab expression library. ScFv molecules are known in the art and are described, e.g., in U.S. Pat. No. 5,892,019. Immunoglobulin or antibody molecules encompassed by this disclosure can be of any

type (e.g., IgG, IgE, IgM, IgD, IgA, and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. Also contemplated are immunoglobulin new antigen receptor (IgNAR) isotypes that are bivalent and comprise a single chain that includes an IgNAR 5 variable domain (VNAR). (See, Walsh et al., Virology 411: 132-141, 2011).

By "specifically binds," it is generally meant that a binding molecule, e.g., an antibody or fragment, variant, or derivative thereof binds to an epitope via its antigen binding 10 domain, and that the binding entails some complementarity between the antigen binding domain and the epitope. According to this definition, a binding molecule is said to "specifically bind" to an epitope when it binds to that epitope, via its antigen binding domain more readily than it 15 would bind to a random, unrelated epitope. The term "specificity" is used herein to qualify the relative affinity by which a certain binding molecule binds to a certain epitope. For example, binding molecule "A" can be deemed to have a higher specificity for a given epitope than binding molecule 20 "B," or binding molecule "A" can be said to bind to epitope "C" with a higher specificity than it has for related epitope

As used herein, the term "affinity" refers to a measure of the strength of the binding of an individual epitope with one 25 or more antigen binding domains, e.g., of an immunoglobulin molecule. See, e.g., Harlow et al., Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988) at pages 27-28. As used herein, the term "avidity" refers to the overall stability of the complex between a 30 population of antigen binding domains and an antigen. See, e.g., Harlow at pages 29-34. Avidity is related to both the affinity of individual antigen binding domains in the population with specific epitopes, and also the valencies of the immunoglobulins and the antigen. For example, the inter- 35 action between a bivalent monoclonal antibody and an antigen with a highly repeating epitope structure, such as a polymer, would be one of high avidity. An interaction between a between a bivalent monoclonal antibody with a also be of high avidity.

As used herein, the term "heavy chain subunit" or "heavy chain domain" includes amino acid sequences derived from an immunoglobulin heavy chain, a binding molecule, e.g., an antibody comprising a heavy chain subunit can include at 45 least one of: a VH domain, a CH1 domain, a hinge (e.g., upper, middle, and/or lower hinge region) domain, a CH2 domain, a CH3 domain, a CH4 domain, or a variant or fragment thereof.

As used herein, the term "light chain subunit" or "light 50 chain domain" includes amino acid sequences derived from an immunoglobulin light chain. The light chain subunit includes at least one of a VL or CL (e.g., Cκ or Cλ) domain.

Binding molecules, e.g., antibodies or antigen binding fragments, variants, or derivatives thereof can be described 55 or specified in terms of the epitope(s) or portion(s) of an antigen that they recognize or specifically bind. The portion of a target antigen that specifically interacts with the antigen binding domain of an antibody is an "epitope," or an "antigenic determinant." A target antigen can comprise a 60 single epitope or at least two epitopes, and can include any number of epitopes, depending on the size, conformation, and type of antigen.

As used herein, the terms "linked," "fused" or "fusion" or other grammatical equivalents can be used interchangeably. 65 These terms refer to the joining together of two more elements or components, by whatever means including

chemical conjugation or recombinant means. An "in-frame fusion" refers to the joining of two or more polynucleotide open reading frames (ORFs) to form a continuous longer ORF, in a manner that maintains the translational reading frame of the original ORFs. Thus, a recombinant fusion protein is a single protein containing two or more segments that correspond to polypeptides encoded by the original ORFs (which segments are not normally so joined in nature). Although the reading frame is thus made continuous throughout the fused segments, the segments can be physically or spatially separated by, for example, in-frame linker sequence. For example, polynucleotides encoding an IMP and a vaccinia virus EEV-specific protein can be fused, in-frame, but be separated by a polynucleotide encoding a linker or spacer, as long as the "fused" open reading frames are co-translated as part of a continuous polypeptide.

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As used herein, the term "hemagglutinin tag" or "HA tag" is a protein derived from a human influenza hemagglutinin surface glycoprotein (HA) corresponding to amino acids 98-106. The HA tag is extensively used as a general epitope tag in expression vectors. Recombinant proteins can be engineered to express the HA tag, which does not appear to interfere with the bioactivity or the biodistribution of the recombinant protein. This tag facilitates the detection, isolation, and purification of the protein of interest.

In the context of polypeptides, a "linear sequence" or a "sequence" is an order of amino acids in a polypeptide from the amino or N-terminus to the carboxyl or C-terminus, in which amino acids that neighbor each other in the sequence are contiguous in the primary structure of the polypeptide.

A portion of a polypeptide that is "amino-terminal" or "N-terminal" to another portion of a polypeptide is that portion that comes earlier in the sequential polypeptide chain. Similarly, a portion of a polypeptide that is "carboxyterminal" or "C-terminal" to another portion of a polypeptide is that portion that comes later in the sequential polypeptide chain.

The term "expression" as used herein refers to a process receptor present at a high density on a cell surface would 40 by which a gene produces a biochemical, for example, a polypeptide. The process includes any manifestation of the functional presence of the gene within the cell including, without limitation, gene knockdown as well as both transient expression and stable expression. It includes without limitation transcription of the gene into messenger RNA (mRNA), and the translation of such mRNA into polypeptide(s). If the final desired product is a biochemical, expression includes the creation of that biochemical and any precursors. Expression of a gene produces a "gene product." As used herein, a gene product can be either a nucleic acid, e.g., a messenger RNA produced by transcription of a gene, or a polypeptide that is translated from a transcript. Gene products described herein further include nucleic acids with post transcriptional modifications, e.g., polyadenylation, or polypeptides with post translational modifications, e.g., methylation, glycosylation, the addition of lipids, association with other protein subunits, proteolytic cleavage, and the like.

> The term "eukaryote" or "eukaryotic organism" is intended to encompass all organisms in the animal, plant, and protist kingdoms, including protozoa, fungi, yeasts, green algae, single celled plants, multi celled plants, and all animals, both vertebrates and invertebrates. The term does not encompass bacteria or viruses. A "eukaryotic cell" is intended to encompass a singular "eukaryotic cell" as well as plural "eukaryotic cells," and comprises cells derived from a eukaryote.

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The term "vertebrate" is intended to encompass a singular "vertebrate" as well as plural "vertebrates," and comprises mammals and birds, as well as fish, reptiles, and amphibians.

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The term "mammal" is intended to encompass a singular "mammal" and plural "mammals," and includes, but is not 5 limited to humans; primates such as apes, monkeys, orangutans, and chimpanzees; canids such as dogs and wolves; felids such as cats, lions, and tigers; equids such as horses, donkeys, and zebras, food animals such as cows, pigs, and sheep; ungulates such as deer and giraffes; rodents such as mice, rats, hamsters and guinea pigs; and bears. In certain aspects, the mammal is a human subject.

The terms "tissue culture" or "cell culture" or "culture" or "culturing" refer to the maintenance or growth of plant or animal tissue or cells in vitro under conditions that allow 15 preservation of cell architecture, preservation of cell function, further differentiation, or all three. "Primary tissue cells" are those taken directly from tissue, i.e., a population of cells of the same kind performing the same function in an organism. Treating such tissue cells with the proteolytic 20 enzyme trypsin, for example, dissociates them into individual primary tissue cells that grow or maintain cell architecture when seeded onto culture plates. Cell cultures arising from multiplication of primary cells in tissue culture are called "secondary cell cultures." Most secondary cells 25 divide a finite number of times and then die. A few secondary cells, however, can pass through this "crisis period," after which they are able to multiply indefinitely to form a continuous "cell line." The liquid medium in which cells are cultured is referred to herein as "culture medium" or "cul-30" ture media." Culture medium into which desired molecules, e.g., viruses or proteins, e.g., immunoglobulin molecules, have been secreted during culture of the cells therein can be referred to as "conditioned medium."

As used herein, the term "identify" refers to methods in which a desired molecule, e.g., a polynucleotide encoding a protein of interest with a desired characteristics or function, is differentiated from a plurality or library of such molecules. Identification methods include "selection" and "screening" or "panning." As used herein, "selection" methods are those in which the desired molecules can be directly separated from the library, e.g., via drug resistance. As used herein, "screening" or "panning" methods are those in which the desired molecules are subjected to an assay in which the desired molecule can be detected. Aliquots of the pools in which the molecule is detected are then divided into successively smaller pools which are likewise assayed, until a pool which is highly enriched from the desired molecule is achieved.

Press, p. 2090 (1990).

Suitable poxvirus virus, e.g., (Mayr, A. et al., Infectorium of the virus, produces four interacellular enveloped enveloped virion (CE) virion (EEV). The presingle lipoprotein mendoth by the desired molecule is achieved.

Poxviruses, E.g., Vaccinia Virus EEV Vectors

IMP fusion proteins as provided herein are produced in poxvirus vectors, e.g., vaccinia virus vectors. The term "poxvirus" includes any member of the family Poxviridae. 55 See, for example, B. Moss in: Virology, 2d Edition, B. N. Fields, D. M. Knipe et al., Eds., Raven Press, p. 2080 (1990). The genus of orthopoxvirus includes, e.g., vaccinia virus, variola virus (the virus that causes smallpox), and raccoon poxvirus. Vaccinia virus is the prototype orthopoxvirus and 60 has been developed and is well-characterized as a vector for the expression of heterologous proteins.

In those embodiments where poxvirus vectors, in particular vaccinia virus vectors, are used to express IMP fusion proteins as provided herein, any suitable poxvirus vector can 65 be used. In certain aspects, the location of a gene encoding an IMP fusion protein can be in a region of the vector that

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is non-essential for growth and replication of the virus so that infectious viruses are produced. Although a variety of non-essential regions of the vaccinia virus genome have been characterized, the most widely used locus for insertion of foreign genes is the thymidine kinase locus, located in the HindIII J fragment in the genome. In certain vaccinia virus vectors, the tk locus has been engineered to contain one or two unique restriction enzyme sites, allowing for convenient use of the trimolecular recombination method recombinant virus production, as described elsewhere herein.

Polynucleotides encoding IMP fusion proteins as provided herein can be inserted into poxvirus vectors, particularly vaccinia virus vectors, under operable association with a transcriptional control region which functions in the cytoplasm of a poxvirus-infected cell.

Poxvirus transcriptional control regions comprise a promoter and a transcription termination signal. Gene expression in poxviruses is temporally regulated, and promoters for early, intermediate, and late genes possess varying structures. Certain poxvirus genes are expressed constitutively, and promoters for these "early-late" genes bear hybrid structures. Synthetic early-late promoters have also been developed. Suitable poxvirus promoters for expressing IMP fusion proteins as provided herein include, but are not limited to late promoters such as the 7.5-kD promoter, the MIL promoter, the 37-kD promoter, the 11-kD promoter, the 11L promoter, the 12L promoter, the 13L promoter, the 15L promoter, the 17L promoter, the 28-kD promoter, the H1L promoter, the H3L promoter, the H5L promoter, the H6L promoter, the H8L promoter, the D11L promoter, the D12L promoter, the D13L promoter, the A1L promoter, the A2L promoter, the A3L promoter, and the P4b promoter. See, e.g., Moss, B., "Poxviridae and their Replication" IN Virology, 2d Edition, B. N. Fields, D. M. Knipe et al., Eds., Raven

Suitable poxvirus vectors include wild-type vaccinia virus, e.g., strain Western Reserve or WR, or attenuated vaccinia virus, e.g., modified vaccinia Ankara (MVA) (Mayr, A. et al., Infection 3:6-14 (1975)).

During its replication cycle, a poxvirus, e.g., a vaccinia virus, produces four infectious forms which differ in their membrane structure: intracellular mature virion (IMV), the intracellular enveloped virion (IEV), the cell-associated enveloped virion (CEV) and the extracellular enveloped virion (EEV). The prevailing view is that the IMV have a single lipoprotein membrane, while the CEV and EEV are both surrounded by two membrane layers and the IEV has three envelopes. EEV is shed from the plasma membrane of the host cell and the EEV membrane is derived from the 50 trans-Golgi.

After infection, the virus loses its membrane(s) and the DNA/protein core is transported along microtubules into the cell. The proteins encoded by early vaccinia mRNAs ("early" is defined as pre-DNA replication) lead to uncoating of the vaccinia core and subsequent DNA replication. This replication occurs in what are termed "viral factories" which are located essentially on top of the ER. Within the viral factory, immature virions (IV) assemble and are processed to form IMV (Intracellular Mature Virus). IMVs contain a membrane that is derived from the ER. The majority of IMVs are released from the cell by cell lysis. Some IMVs are transported on microtubules to sites of wrapping by membranes of the trans-Golgi network or early endosomes. The wrapping of the IMV particles by a double membrane creates a form of vaccinia called IEVs (Intracellular Enveloped Virus). The IEVs are then transported to the cell surface on microtubules. The outer IEV membrane fuses

with the plasma membrane to expose a CEV (Cell Associated Enveloped Virus) at the cell surface. Actin polymerization from the host cell can drive the CEV to infect neighboring cells, or the virus can be released as an EEV. See, e.g., Kim L. Roberts and Geoffrey L. Smith. Trends in Microbiology 16 (10):472-479 (2008); Geoffrey L. Smith, et al., Journal of General Virology 83:2915-2931 (2002).

At least six virus-encoded proteins have been reported as $components \ of \ the \ EEV \ envelope \ membrane. \ Of these, four \ _{10} \ \ _{DETPEPITDKEDHTVTDTVSYTTVSTSSGIVTTKSTTDDADLYDTYNDNDTSSGIVTTKSTTDDADLYDTYNDNTSSGIVTTKSTTDDADLYDTYNDNTSSGIVTTKSTTDDADLYDTYNDNTSSGIVTTKSTTDDADLYDTYNDNTSSGIVTTKSTTDDADLYDTYNDNTSSGIVTTKSTTDDADLYDTYNDNTSSGIVTTKSTTTTNTSSGIVTTKSTTTNTSSGIVTTKSTTTNTSSGIVTTKSTTTNTSSGIVTTKSTTTNTSSGIVTTKSTTTNTSSGIVTTKSTTTNTSSGIVTTKSTTTNTSSGIVTTKSTTTNTSSGIVTTKSTTTNTSSGIVTTKSTTTNTSSGIVTTKSTTTNTSSGIVTTKSTTTNTSSGIVTTKSTTTNTSSGIVTTKSTTNTSSGIVTTKSTTNTSSGIVTTKSTTNTSSGIVTTKSTTNTSSGIVTTKSTTNTSSGIVTTKSTTNTSSGIVTTKSTTTNTSSGIVTTTNTSSGIVTTKSTTNTSSGIVTTTNTSSGIVTTTTTNTSSGIVTTT$ proteins (A33R, A34R, A56R, and B5R) are glycoproteins, one (A36R) is a nonglycosylated transmembrane protein, and one (F13L) is a palmitovlated peripheral membrane protein. See, e.g., Lorenzo et al., Journal of Virology 74 (22):10535 (2000). During infection, these proteins localize 15 to the Golgi complex, where they are incorporated into infectious virus that is then transported and released into the extracellular medium. As provided herein, IMP fusion proteins are directed to and expressed on the EEV membrane as a fusion protein with an EEV-specific protein, e.g., F13L or 20

The F13L protein is associated with the interior surface of the outermost EEV membrane through palmitoylation of cysteines 185 and 186. Smith Trends in Microbiol. 16:472- 25 479 (2008). Vaccinia viruses in which the gene encoding F13L is deleted form tiny plaques and the number of EEV produced is reduced significantly.

The amino acid sequence of the F13L protein from vaccinia virus strain WR is presented as SEQ ID NO: 1. The 30 two palmitoylated cysteine residues (amino acids 85 and 86 of SEQ ID NO: 1) are underlined. Since F13L does not cross the membrane, it does not have a transmembrane domain or signal peptide.

>F13L (SEQ ID NO: 1) MWPFASVPAGAKCRLVETLPENMDFRSDHLTTFECFNEIITLAKKYIYIAS FCCNPLSTTRGALIFDKLKEASEKGIKIIVLLDERGKRNLGELQSHCPDIN FITVNIDKKNNVGLLLGCFWVSDDERCYVGNASFTGGSIHTIKTLGVYSDY PPLATDLRRRFDTFKAFNSAKNSWLNLCSAACCLPVSTAYHIKNPIGGVFF TDSPEHLLGYSRDLDTDVVIDKLKSAKTSIDIEHLAIVPTTRVDGNSYYWP DIYNSIIEAAINRGVKIRLLVGNWDKNDVYSMATARSLDALCVQNDLSVKV ${\tt FTIQNNTKLLIVDDEYVHITSANFDGTHYQNHGFVSFNSIDKQLVSEAKKI}$ FERDWVSSHSKSLKI

The A56R protein is the vaccinia virus hemagglutinin and is a standard type I integral membrane protein comprising an amino-terminal extracellular ("extra-membrane") domain, a single transmembrane domain, and a cytoplasmic ("intramembrane") domain. A56R comprises an N-terminal signal peptide of about 33 amino acids, an Ig-like domain extending from about amino acid 34 to about amino acid 103, a stalk region extending from about amino acid 121 to about amino acid 275, a transmembrane domain extending from about amino acid 276 to about amino acid 303, and an cytoplasmic ("inter-membrane") domain extending from about amino acid 304 to amino acid 314. See DeHaven et al., 65 J. Gen Virol. 92:1971-1980 (2011). A56R is presented as SEQ ID NO: 5.

>A56R

(SEQ ID NO: 5)

MTRLPILLLISLVYATPFPQTSKKIGDDATLSCNRNNTNDYVVMSAWYKE

20

PNSIILLAAKSDVLYFDNYTKDKISYDSPYDDLVTTITIKSLTARDAGTYV

CAFFMTSTTNDTDKVDYEEYSTELIVNTDSESTIDIILSGSTHSPETSSKK

PDYIDNSNCSSVFEIATPEPITDNVEDHTDTVTYTSDSINTVSASSGESTT

VPPTTVGGSTTSISNYKTKDFVEIFGITALIILSAVAIFCITYYIYNKRSR

KYKTENKV

IMP fusion proteins as provided herein can be expressed in any suitable vaccinia virus. In certain embodiments, the DNA encoding an EEV fusion protein can be inserted into a region of the vaccinia virus genome which is non-essential for growth and replication of the vector so that infectious viruses are produced. Although a variety of non-essential regions of the vaccinia virus genome have been characterized, the most widely used locus for insertion of foreign genes is the thymidine kinase locus, located in the HindIII J fragment in the genome. IMP fusion proteins as provided herein can be inserted into vaccinia virus vectors under operable association with a transcriptional control region which functions in the cytoplasm of a poxvirus-infected cell.

Suitable promoters for use in the methods described herein include, without limitation, the early/late 7.5-kD promoter, or the early/late H5 promoter (or variants thereof).

The Tri-Molecular Recombination Method

Tri-molecular recombination, as disclosed in Zauderer, 35 PCT Publication No. WO 00/028016 and in U.S. Pat. No. 7,858,559, is a high efficiency, high titer-producing method for expressing proteins of interest and or producing libraries in vaccinia virus. The tri-molecular recombination method allows the generation of recombinant viruses at efficiencies of at least 90%, and titers at least at least 2 orders of magnitude higher than those obtained by direct ligation.

In certain aspects, IMP fusion proteins for expression in vaccinia virus and display on EEV as described herein can be constructed in poxvirus vectors, e.g., vaccinia virus vectors, by tri-molecular recombination.

In certain embodiments, a transfer plasmid for IMP fusion proteins for expression in EEV is provided, which comprises a polynucleotide flanking regions in the vaccinia virus Tk gene, the vaccinia virus H5 promoter, and NcoI and BsiWI restriction sites for inserting coding regions for desired fusion proteins.

Integral Membrane Proteins

The disclosure provides a method for expressing integral membrane proteins (IMPs) in a conformationally intact state that approaches the native conformation of the protein as it would appear in a cell in which the protein is naturally expressed. According to the disclosure, IMPs are expressed as fusion proteins with poxvirus proteins that are expressed on poxvirus, e.g., vaccinia virus EEVs. IMP fusion proteins as provided herein, when expressed and displayed on the surface of EEVs, are useful as target antigens for screening libraries of binding molecules, e.g., antibody display librar-

Any IMP can be constructed as a fusion protein according to the methods provided herein. In certain aspects the IMP

is a target for immunotherapy. In certain aspects the IMP is a multi-pass IMP such as CD20 or a G-protein coupled receptor (GPCR). Suitable multi-pass human IMPs for use

in the construction of IMP fusion proteins as provided herein include, without limitation, the proteins listed in Table 1.

TABLE 1

		ENTREZ gene	# predicted TM
Protein Name	ENTREZ_gene_ID	symbol	domains
Poliovirus receptor-related protein 3	25945	PVRL3	2
Prominin-1	8842	PROM1	5
FL cytokine receptor	2322	FLT3	2
Scavenger receptor cysteine-rich type 1	9332	CD163	2
protein M130	2577	077.071	
C-X-C chemokine receptor type 1 C-X-C chemokine receptor type 3	3577 2833	CXCR1	6 7
C-X-C chemokine receptor type 5	2833 643	CXCR3 CXCR5	7
C-C chemokine receptor type 4	1233	CCR4	7
C-C chemokine receptor type 7	1236	CCR7	7
B-lymphocyte antigen CD20	931	MS4A1	4
Major prion protein	5621	PRNP	2
Plexin-C1	10154	PLXNC1	2
Multidrug resistance protein 1	5243	ABCB1	12
Putative G-protein coupled receptor 44	11251	GPR44	7
EGF-like module-containing mucin-like	30817	EMR2	7
hormone receptor-like 2 Frizzled-4	8322	FZD4	9
Leukocyte surface antigen CD47	961	CD47	5
CD63 antigen	967	CD63	4
Choline transporter-like protein 1	23446	SLC44A1	9
CD97 antigen	976	CD97	7
Multidrug resistance-associated protein 1	4363	ABCC1	16
CAS1 domain-containing protein 1	64921	CASD1	14
Solute carrier family 12 member 6	9990	SLC12A6	14
Sodium/hydrogen exchanger 1	6548	SLC9A1	13
Solute carrier family 12 member 9	56996	SLC12A9	13
Solute carrier family 2, facilitated glucose	6513	SLC2A1	12
transporter member 1 Sodium- and chloride-dependent taurine transporter	6533	SLC6A6	12
Solute carrier organic anion transporter family member 4A1	28231	SLCO4A1	12
Solute carrier family 23 member 2	9962	SLC23A2	12
Solute carrier organic anion transporter family member 3A1	28232	SLCO3A1	12
Prestin	375611	SLC26A5	11
Equilibrative nucleoside transporter 2	3177	SLC29A2	11
Equilibrative nucleoside transporter 1	2030	SLC29A1	11
Sodium-coupled neutral amino acid transporter 1	81539	SLC38A1	11
Sodium bicarbonate cotransporter 3	9497	SLC4A7	11
Urea transporter 1	6563	SLC14A1	10
Transmembrane and coiled-coil domain-containing protein 3	55002	TMCO3	10
Signal peptide peptidase-like 2A	84888	SPPL2A	9
Transmembrane 9 superfamily member 3	56889	TM9SF3	9
Anoctamin-9	338440	ANO9	8
Sodium/potassium-transporting ATPase subunit alpha-1	476	ATP1A1	8
Sodium/potassium-transporting ATPase subunit alpha-3	478	ATP1A3	8
Anoctamin-6 V-type proton ATPase 116 kDa subunit a	196527 23545	ANO6 ATP6V0A2	8 8
isoform 2	27224	DODATA	_
Putative P2Y purinoceptor 10	27334	P2RY10	7
G-protein coupled receptor 39	2863	GPR39	7
Sphingosine 1-phosphate receptor 2 Latrophilin-2	9294	S1PR2	7 7
Beta-2 adrenergic receptor	23266 154	LPHN2 ADRB2	7
Alpha-2C adrenergic receptor	152	ADRB2 ADRA2C	7
Thromboxane A2 receptor	6915	TBXA2R	7
Platelet-activating factor receptor	5724	PTAFR	7
Proteinase-activated receptor 1	2149	F2R	7
Neuropeptide Y receptor type 1	4886	NPY1R	7
Type-1 angiotensin II receptor	185	AGTR1	7
Neurotensin receptor type 1	4923	NTSR1	7
Cannabinoid receptor 2	1269	CNR2	7
Camiadinoid receptor 2			

TABLE 1-continued

IABLE 1-continued					
Exemplary Human M	Exemplary Human Multi-Pass Integral Membrane Proteins				
Protein Name	ENTREZ_gene_ID	ENTREZ gene symbol	# predicted TM domains		
Calcitonin gene-related peptide type 1 receptor	10203	CALCRL	7		
Protein GPR107	57720	GPR107	7		
G-protein coupled receptor 126	57211	GPR126	7		
P2Y purinoceptor 8	286530	P2RY8	7		
Probable G-protein coupled receptor 125	166647	GPR125	7		
Transmembrane protein 87A Mas-related G-protein coupled receptor	25963 116535	TMEM87A MRGPRF	7 7		
member F	110555	MIKGFKI	,		
Transmembrane protein 87B	84910	TMEM87B	7		
Proteinase-activated receptor 4	9002	F2RL3	7		
Smoothened homolog	6608	SMO	7		
EGF-like module-containing mucin-like hormone receptor-like 3	84658	EMR3	7		
Nemomedin-U receptor 1	10316	NMUR1	7		
EGF, latrophilin and seven transmembrane	64123	ELTD1	7		
domain-containing protein 1					
Transmembrane protein 8A	58986	TMEM8A	7		
Cadherin EGF LAG seven-pass G-type	1952	CELSR2	7		
receptor 2	0.620	CEL CD1	7		
Cadherin EGF LAG seven-pass G-type receptor 1	9620	CELSR1	7		
Cadherin EGF LAG seven-pass G-type	1951	CELSR3	7		
receptor 3					
Cysteinyl leukotriene receptor 1	10800	CYSLTR1	7		
G-protein coupled receptor 56	9289	GPR56	7		
Lipid phosphate phosphohydrolase 1 Potassium voltage-gated channel subfamily A	8611 3738	PPAP2A KCNA3	6 6		
member 3	3736	KCNAS	O		
Zinc transporter ZIP6	25800	SLC39A6	6		
Zinc transporter ZIP14	23516	SLC39A14	6		
P2Y purinoceptor 11	5032	P2RY11	6		
Zinc transporter ZIP10	57181	SLC39A10	6		
Cytochrome b-245 heavy chain	1536	CYBB	5 5		
Prominin-2 Protein tweety homolog 2	150696 94015	PROM2 TTYH2	5		
Protein tweety homolog 3	80727	TTYH3	5		
Gamma-aminobutyric acid receptor subunit	2562	GABRB3	4		
beta-3					
Glutamate receptor, ionotropic kainate 3	2899	GRIK3	4		
Neuronal membrane glycoprotein M6-b	2824	GPM6B	4		
Metal transporter CNNM4 Metal transporter CNNM3	26504 26505	CNNM4 CNNM3	4 3		
Discoidin, CUB and LCCL domain-	131566	DCBLD2	3		
containing protein 2					
Transmembrane protein 131-like	23240	KIAA0922	2		
Leucine-rich repeat transmembrane protein	23768	FLRT2	2		
FLRT2	0.455	ATDAT	2		
Attractin Receptor-type tyrosine-protein phosphatase	8455 5793	ATRN PTPRG	2 2		
gamma	3173	TITIKO	2		
Interferon alpha/beta receptor 2	3455	IFNAR2	2		
Ephrin type-A receptor 5	2044	EPHA5	2		
Tyrosine-protein kinase transmembrane	4919	ROR1	2		
receptor ROR1	0.577	TM APPEA	2		
Tomoregulin-1 P2X purinoceptor 7	8577 5027	TMEFF1 P2RX7	2 2		
TM2 domain-containing protein 3	80213	TM2D3	2		
TM2 domain-containing protein 1	83941	TM2D3	2		
G-protein coupled receptor 64	10149	GPR64	8		
Psychosine receptor	8477	GPR65	6		
Large neutral amino acids transporter small	8140	SLC7A5	12		
subunit 1	1002	CIDDO	7		
Sphingosine 1-phosphate receptor 3 Solute carrier organic anion transporter	1903 6578	S1PR3 SLCO2A1	12		
family member 2A1	0370	blc02A1	12		
Type-2 angiotensin II receptor	186	AGTR2	7		
UPF0513 transmembrane protein	79583	UNQ870/PRO1886	2		
Lipid phosphate phosphohydrolase 3	8613	PPAP2B	5		
Blood vessel epicardial substance	11149	BVES	3		
Sodium/potassium/calcium exchanger 6	80024	SLC24A6	13		
5-hydroxytryptamine receptor 2B Mucolipin-1	3357 57192	HTR2B MCOLN1	7 6		
Cadherin-8	1006	CDH8	2		
Adenosine receptor A1	134	ADORA1	7		
	151	51411	,		

TABLE 1-continued

Exemplary Human Multi-Pass Integral Membrane Proteins				
Protein Name	ENTREZ_gene_ID	ENTREZ gene symbol	# predicted TM domains	
Probable G-protein coupled receptor 110	266977	GPR110	7	
Chemokine receptor-like 1	1240	CMKLR1	7	
Proton-coupled folate transporter	113235	SLC46A1	11	
Sphingosine 1-phosphate receptor 4 Protein FAM171A2	8698 284069	S1PR4 FAM171A2	7 2	
Alpha-2A adrenergic receptor	150	ADRA2A	7	
C-X-C chemokine receptor type 7	57007	CXCR7	7	
Apelin receptor	187	APLNR	7	
Probable G-protein coupled receptor 116	221395	GPR116	7	
Metalloreductase STEAP4	79689	STEAP4	6	
Solute carrier organic anion transporter family member 4C1	353189	SLCO4C1	12	
ATP-binding cassette sub-family A member 8	10351	ABCA8	14	
Vasoactive intestinal polypeptide receptor 1	7433	VIPR1	7	
SID1 transmembrane family member 2 Equilibrative nucleoside transporter 4	51092 222962	SIDT2 SLC29A4	11 10	
Succinate receptor 1	56670	SLC29A4 SUCNR1	7	
Metal transporter CNNM2	54805	CNNM2	4	
Probable palmitoyltransferase ZDHHC5	25921	ZDHHC5	4	
Solute carrier family 22 member 16	85413	SLC22A16	12	
Leukotriene B4 receptor 1	1241	LTB4R	7	
Pannexin-1	24145	PANX1	4	
Sodium-dependent glucose transporter 1	91749	NAGLT1	11	
Sodium/calcium exchanger 1	6546	SLC8A1	10	
Neuronal acetylcholine receptor subunit alpha-3	1136	CHRNA3	4	
Retinoic acid-induced protein 3	9052	GPRC5A	7	
Lysophosphatidic acid receptor 5	57121	LPAR5	7	
Probable G-protein coupled receptor 132	29933	GPR132	7	
Sphingosine 1-phosphate receptor 5	53637	S1PR5	7 7	
Endothelin-1 receptor Probable G-protein coupled receptor 124	1909 25960	EDNRA GPR124	7	
Solute carrier family 12 member 7	10723	SLC12A7	12	
Thyrotropin receptor	7253	TSHR	7	
Transient receptor potential cation channel subfamily V member 2	51393	TRPV2	6	
Glutamate receptor delta-1 subunit	2894	GRID1	4	
Gamma-aminobutyric acid receptor subunit alpha-2	2555	GABRA2	4	
Sphingosine 1-phosphate receptor 1	1901	S1PR1	7	
Prostaglandin E2 receptor EP3 subtype	5733	PTGER3	7	
Probable G-protein coupled receptor 174	84636	GPR174	7	
Glutamate receptor 2	2891	GRIA2	3	
Amiloride-sensitive sodium channel subunit delta	6339	SCNN1D	2	
5-hydroxytryptamine receptor 1D	3352	HTR1D	7	
Goliath homolog	55819	RNF130	2	
ATP-binding cassette sub-family A member 7 Prostacyclin receptor	10347 5739	ABCA7 PTGIR	11 7	
Probable G-protein coupled receptor 176	11245	GPR176	7	
Thyrotropin-releasing hormone receptor	7201	TRHR	7	
Claudin-12	9069	CLDN12	4	
Protein FAM38A	9780	FAM38A	29	
Niemann-Pick C1 protein	4864	NPC1	13	
Synaptic vesicle glycoprotein 2A	9900	SV2A	12	
Signal peptide peptidase-like 2B	56928	SPPL2B	9	
Rhomboid family member 2	79651	RHBDF2	7	
Immunoglobulin superfamily member 1 Dolichyl-diphosphooligosaccharideprotein	3547 6185	IGSF1 RPN2	4 3	
glycosyltransferase subunit 2 Transmembrane emp24 domain-containing	54732	TMED9	2	
protein 9	410	ama	2	
Steryl-sulfatase	412	STS	2	
Transmembrane 9 superfamily member 1	10548	TM9SF1	9	
Melanoma inhibitory activity protein 3 Arylsulfatase F	375056 416	MIA3 ARSF	2 2	
Solute carrier family 2, facilitated glucose	6517	SLC2A4	12	
transporter member 4	0317	SECZAT	12	
Anoctamin-5	203859	ANO5	8	
Nicalin	56926	NCLN	2	

In certain aspects, the multi-pass IMP is a GPCR, e.g., FZD4 or CXCR4. In certain aspects the multi-pass IMP is CD20.

Polynucleotides Encoding IMP Fusion Proteins for Expression on Poxvirus EEV

This disclosure provides an isolated polynucleotide for expression of an integral membrane protein or fragment thereof in a conformationally-intact form in the context of a 10 biological membrane, as a fusion with a protein or fragment thereof specific for vaccinia virus EEV. By "conformationally intact" is meant that the protein appears, or is displayed, in a native or close to native conformation in the context of 15 a biological lipid bilayer membrane, much as the protein would appear in its native state.

In one aspect, the disclosure provides an isolated polynucleotide that includes a first nucleic acid fragment that encodes an integral membrane protein (IMP) or fragment 20 thereof, e.g., a multi-pass IMP, where the IMP or fragment thereof comprises at least one extra-membrane region, at least one transmembrane domain and at least one intramembrane region, and where a portion of the first nucleic acid fragment encoding at least one intra-membrane region 25 is situated at the 5' or 3' end of the first nucleic acid fragment; and a second nucleic acid fragment that encodes a vaccinia virus F13L protein (SEQ ID NO: 1) or functional fragment thereof, where the second nucleic acid fragment is fused in frame to a portion of the first nucleic acid fragment that 30 encodes an intra-membrane region of the IMP. The first nucleic acid fragment and the second nucleic acid fragment can, in some instances, we separated by a nucleic acid encoding a linker or other spacer. The polynucleotide can further include a poxvirus promoter operably associated 35 LAKKYIYIASFCCNPLSTTRGALIFDKLKEASEKGIKIIVLLDERGKRNLG with the first and second nucleic acid fragments, allowing expression of the polynucleotide in the cytoplasm of a poxvirus-infected cell. According to this aspect, a poxvirusinfected cell that contains the polynucleotide can express an IMP-F13L fusion protein as part of the outer envelope 40 IKNPIGGVFFTDSPEHLLGYSRDLDTDVVIDKLKSAKTSIDIEHLAIVPTT membrane of an extracellular enveloped virion (EEV). Schematic diagrams showing expression of an IMP as a fusion with F13L are shown in FIG. 1B and FIG. 1C.

In certain aspects, the IMP or fragment thereof can be a multi-pass membrane protein comprising at least two, at 45 least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten, at least eleven, at least twelve, or even more transmembrane (TM) domains, such as those listed in Table 1.

Where the IMP has an odd number of TM domains, one 50 end of the IMP, either the N-terminus or the C-terminus, will be naturally situated on the extra-membrane side of the biological membrane and the other end of the IMP will be situated on the intra-membrane side of the IMP. Since the F13L protein is wholly-internal to the outer membrane of 55 poxvirus EEVs, the end of the IMP, the N-terminus or the C-terminus that is situated internal to the membrane can be fused to F13L. Thus for an IMP such as a typical 7-TM domain GPCR in which the N-terminus of the protein is extra-membrane and the C-terminus is intra-membrane, the 60 N-terminus of F13L can be fused to the C-terminus of the GPCR as shown in FIG. 1B. Accordingly, a polynucleotide as above is provided where the first nucleic acid fragment encodes an IMP with an odd number of transmembrane domains, where the 5' end of the first nucleic acid fragment 65 encodes the extra-membrane region, and the 3' end of the first nucleic acid fragment encodes the intra-membrane

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region of the IMP, the latter being fused to the 5' end of the nucleic acid fragment encoding F13L or a fragment thereof.

In an exemplary polynucleotide of this type, the first polynucleotide can encode the human frizzled-4 protein (FZD4), or a fragment thereof, a target for immunotherapy of certain human cancers, fused to the N-terminus of F13L. Accordingly, a polynucleotide which encodes an FZD4-F13L fusion protein is provided. An exemplary polynucleotide according to this aspect encodes the mature fusion protein, amino acids 20 to 892 of SEQ ID NO: 2, as shown below. The polynucleotide can further encode a signal peptide, e.g., the signal peptide of FZD4, amino acids 1 to 19 of SEQ ID NO: 2.

FZD (FL)-F13L

(SEQ ID NO: 2)

MGWSCIILFLVATATGAHS FGDEEERRCDPIRISMCQNLGYNVTKMPNLVG HELQTDAELQLTTFTPLIQYGCSSQLQFFLCSVYVPMCTEKINIPIGPCGG MCLSVKRRCEPVLKEFGFAWPESLNCSKFPPQNDHNHMCMEGPGDEEVPLP HKTPIQPGEECHSVGTNSDQYIWVKRSLNCVLKCGYDAGLYSRSAKEFTDI WMAVWASLCFISTAFTVLTFLIDSSRFSYPERPIIFLSMCYNIYSIAYIVR LTVGRERISCDFEEAAEPVLIQEGLKNTGCAIIFLLMYFFGMASSIWWVIL TLTWFLAAGLKWGHEAIEMHSSYFHIAAWAIPAVKTIVILIMRLVDADELT ${\tt GLCYVGNQNLDALTGFVVAPLFTYLVIGTLFIAAGLVALFKIRSNLQKDGT}$ KTDKLERLMVKIGVFSVLYTVPATCVIACYFYEISNWALFRYSADDSNMAV EMLKIFMSLLVGITSGMWIWSAKTLHTWQKCSNRLVNSGKVKREKRGNGWV KPGKGSETVVMWPFASVPAGAKCRLVETLPENMDFRSDHLTTFECFNEIIT ELOSHCPDINFITVNIDKKNNVGLLLGCFWVSDDERCYVGNASFTGGSIHT TKTLGVYSDYPPLATDLRRRFDTFKAFNSAKNSWLNLCSAACCLPVSTAYH RVDGNSYYWPDIYNSIIEAAINRGVKIRLLVGNWDKNDVYSMATARSLDAL CVONDLSVKVFTIONNTKLLIVDDEYVHITSANFDGTHYONHGFVSFNSID

KOLVSEAKKIFERDWVSSHSKSLKI Single Underline - leader peptide (amino acids 1-19)
Bold - human Fzd4 (amino acids 20-520) Italics - F13L (amino acids 521-892)

In another exemplary polynucleotide of this type, the first polynucleotide can encode A CXC chemokine receptor, or a fragment thereof, fused to the N-terminus of F13L. CXC chemokine receptors are likewise targets for immunotherapy of certain human cancers. An exemplary CXC chemokine receptor is CXCR4, or a fragment thereof. Accordingly, a polynucleotide which encodes a CXC chemokine receptor-F13L fusion protein, e.g., a CXCR4-F13L fusion protein is provided. An exemplary polynucleotide according to this aspect encodes SEQ ID NO: 3, as shown below.

CXCR4-F13L

(SEQ ID NO: 3)

MAIPLPLLQIYTSDNYTEEMGSGDYDSMKEPCFREENANFNKIFLPTIYSI IFLTGIVGNGLVILVMGYOKKLRSMTDKYRLHLSVADLLFVITLPFWAVDA VANWYFGNFLCKAVHVIYTVNLYSSVLILAFISLDRYLAIVHATNSQRPRK -continued

LLAEKVVYVGVWIPALLLTIPDFIFANVSEADDRYICDRFYPNDLWVVVFQ FQHIMVGLILPGIVILSCYCIIISKLSHSKGHQKRKALKTTVILILAFFAC WLPYYIGISIDSFILLEIIKQGCEFENTVHKWISITEALAFFHCCLNPILY AFLGAKFKTSAOHALTSVSRGSSLKILSKGKRGGHSSVSTESESSSFHSSM WPFASVPAGAKCRLVETLPENMDFRSDHLTTFECFNEIITLAKKYIYIASF CCNPLSTTRGALIFDKLKEASEKGIKIIVLLDERGKRNLGELQSHCPDINFITVNIDKKNNVGLLLGCFWVSDDERCYVGNASFTGGSIHTIKTLGVYSDYP PLATDLRRRFDTFKAFNSAKNSWLNLCSAACCLPVSTAYHIKNPIGGVFFT DSPEHLLGYSRDLDTDVVIDKLKSAKTSIDIEHLAIVPTTRVDGNSYYWPD IYNSIIEAAINRGVKIRLLVGNWDKNDVYSMATARSLDALCVONDLSVKVF TIONNTKLLIVDDEYVHITSANFDGTHYONHGFVSFNSIDKOLVSEAKKIF ERDWVSSHSKSLKI Bold - human CXCR4 (amino acids 1-356) Italics - F13L (amino acids 357-728)

As will be evident to a person of ordinary skill in the art, a multi-pass membrane protein having an even number of $_{25}$ transmembrane domains will be inserted into a biological membrane such that its N-terminus and its C-terminus are on the same side of the membrane, either on the extra-membrane side of the membrane, or on the intra-membrane side of the membrane. Since the F13L protein is situated entirely 30 on the intra-membrane side of poxvirus EEVs, formation of an IMP-F13L fusion protein properly embedded in the membrane would need at least one of the N-terminus or the C-terminus of the IMP or fragment thereof to be internal to the membrane. Where the IMP has an even number of TM 35 domains and both are situated internally, the F13L protein can be fused either to the N-terminus of the IMP or to the C-terminus of the IMP. If the full-length IMP is situated such that both the N- and C-terminus are extra-membrane, a fragment of the IMP having an odd number of TM domains 40 can be fused to F13L.

Accordingly, the disclosure provides a polynucleotide as described above that encodes an IMP with an even number of transmembrane domains, where both the 5' and 3' ends of the first nucleic acid fragment encode intra-membrane 45 regions. In certain aspects the 3' end of the nucleic acid fragment encoding F13L can be fused to the 5' end of the nucleic acid fragment encoding the IMP, in certain aspects the 5' end of the nucleic acid fragment encoding F13L can be fused to the 3' end of the nucleic acid fragment encoding 50

An exemplary IMP of this type is human CD20, a 4-TM domain IMP expressed on human B cells, which is a target for immunotherapy of B cell leukemias, lymphomas, and myelomas. A diagram of a CD20-F13L fusion protein in 55 which the C-terminus of CD20 is fused to the N-terminus of F13L is shown in FIG. 1C. Accordingly, a polynucleotide which encodes a CD20-F13L fusion protein is provided. An exemplary polynucleotide according to this aspect encodes SEQ ID NO: 4, as shown below.

CD20-F13L

(Seq ID NO: 4)

60

MATPRNSVNGTFPAEPMKGPIAMOSGPKPLFRRMSSLVGPTOSFFMRESKT

LGAVQIMNGLFHIALGGLLMIPAGIYAPICVTVWYPLWGGIMYIISGSLLA

-continued

ATEKNSRKCLVKGKMIMNSLSLFAAISGMILSIMDILNIKISHFLKMESLN FIRAHTPYINIYNCEPANPSEKNSPSTQYCYSIQSLFLGILSVMLIFAFFQ ELVIAGIVENEWKRTCSRPKSNIVLLSAEEKKEQTIEIKEEVVGLTETSSQ PKNEEDIEIIPIOEEEEEETETNFPEPPODOESSPIENDSSPMWPFASVPA ${\it GAKCRLVETLPENMDFRSDHLTTFECFNEIITLAKKYIYIASFCCNPLSTT}$ RGALIFDKLKEASEKGIKIIVLLDERGKRNLGELQSHCPDINFITVNIDKK NNVGLLLGCFWVSDDERCYVGNASFTGGSIHTIKTLGVYSDYPPLATDLRR RFDTFKAFNSAKNSWLNLCSAACCLPVSTAYHIKNPIGGVFFTDSPEHLLG YSRDLDTDVVIDKLKSAKTSIDIEHLAIVPTTRVDGNSYYWPDIYNSIIEA AINRGVKIRLLVGNWDKNDVYSMATARSLDALCVONDLSVKVFTIONNTKL LIVDDEYVHITSANFDGTHYONHGFVSFNSIDKOLVSEAKKIFERDWVSSH 20 SKSLKI Bold - human CD20 (MS4A1) (amino acids 1-298) Italics - F13L (amino acids 299-669)

In polynucleotides as provided above, the first and second nucleic acid fragments can be directly fused, or alternatively they can be separated by a nucleic acid fragment encoding a linker or spacer or other polypeptide fragment. In certain aspects, a polynucleotide as provided above can further include a third nucleic acid fragment that encodes a heterologous peptide polypeptide, either between the first and second nucleic acid fragments, or on either side. The heterologous peptide can be, for example, a linker sequence, an amino acid tag or label, or a peptide or polypeptide sequence that facilitates purification. In certain aspects the heterologous peptide is a 6-histidine tag fused, e.g., to the C-terminus of the fusion protein.

In certain aspects, a polynucleotide as provided herein is operably associated with a poxvirus promoter. Suitable promoters are described elsewhere herein. In certain aspects the promoter is a poxvirus p7.5 promoter or a poxvirus H5

A polynucleotide as provided herein can be or can be part of, a poxvirus genome, where the poxvirus genome, upon introduction into a suitable permissive host cell, can produce infectious EEV that display the IMP-F13L fusion protein on their surface. In certain aspects the poxvirus genome is a vaccinia virus genome, e.g., a vaccinia virus WR genome or an MVA genome. A poxvirus genome comprising a polynucleotide as described can be produced by standard molecular biological and virology techniques, for example by using tri-molecular recombination as described herein. A poxvirus genome as provided herein can be introduced into permissive cells as part of a recombinant poxvirus, or as naked DNA accompanied by suitable helper viruses, e.g., fowlpox virus. The disclosure further provides a recombinant poxvirus, e.g., a recombinant vaccinia virus comprising the provided poxvirus genome.

IMP-EEV Fusion Proteins, Recombinant Poxvirus EEVs, and Methods of Making

This disclosure further provides an IMP-F13L fusion protein such as those encoded by the polynucleotides described above. Moreover, the IMP-F13L fusion protein can be expressed on the surface of a recombinant poxvirus EEV, e.g., a recombinant vaccinia virus EEV. A recombinant poxvirus EEV, e.g., a recombinant vaccinia virus EEV

FZD-ECD-A56R

comprising the provided fusion protein is provided by the disclosure. A recombinant poxvirus EEV can be produced by a method that includes infecting a host cell permissive for vaccinia virus infectivity with a vaccinia virus comprising a poxvirus genome as provided above and recovering EEV released from the infected host cell. Accordingly, an IMP-F13L fusion protein encoded by a polynucleotide as described above, is provided.

Moreover the disclosure provides fusion proteins comprising an IMP or fragment thereof, which can be a multipass IMP, and single pass IMP, or even just the extracellular domain (ECD) of the IMP, fused to a poxvirus protein, e.g., a vaccinia virus protein, specific for EEV, such as F13L, A56R, B5R, 33R, A34R, or A36R, an "IMP-EEV fusion protein." Exemplary ECD fusion proteins are described below. An IMP-EEV fusion protein as provided herein can display the IMP, e.g., a multi-pass IMP, single-pass IMP or ECD of an IMP, in a conformationally intact form on the surface of poxvirus EEV. For use in screening antibody display libraries for antigen binding domains that specifically bind to a target IMP, display of IMPs on the surface of poxvirus EEV offers many advantages over displaying IMPs on the surface of recombinant cells, e.g., CHO cells, as is typical. For example, the IMP can be expressed at higher density on EEV than on cells. Moreover, ÊEV express only 25 about six different poxvirus proteins on their surface (e.g., F13L, A56R, B5R, 33R, A34R, and A36R) as opposed to hundreds that might be expressed on the surface of cells. Finally, inactivated EEV expressing IMP-F13L fusion proteins as provided herein can be attached to solid supports, 30 offering convenience in library screening.

Accordingly, this disclosure provides a method to display an integral membrane protein (IMP) or fragment thereof in a native conformation for use, e.g., in screening antibody display libraries for antigen binding domains specific for the 35 IMP. The method includes: infecting host cells permissive for poxvirus infectivity with a recombinant poxvirus that expresses the IMP or fragment thereof as a fusion protein with poxvirus EEV-specific protein or membrane-associated fragment thereof, where EEV produced by the infected host cell comprise the IMP as part of the EEV outer envelope membrane; and recovering EEV released from the host cell. IMP. In certain aspects, the EEV-specific protein or fragment thereof can be the vaccinia virus A33R protein, A34R protein, A56R protein, B5R protein, A36R protein, F13L combination thereof.

In certain aspects, the EEV-specific protein is F13L (SEQ ID NO: 1) or a functional fragment thereof, and the fusion protein can be one expressed by a polynucleotide as proprotein comprising at least two, at least three, at least four, at least five, at least six or at least seven transmembrane

In certain aspects, the membrane-associated EEV specific protein fragment includes the stalk, transmembrane, and $_{55}$ $_{STELIVNTDSESTIDIILSGSTHSPETSSKKPDYIDNSNCSSVFEIATPEP}$ intra-membrane domains of the vaccinia virus A56R protein, a fragment comprising, consisting of, or consisting essentially of amino acids 108 to 314 of SEQ ID NO: 5. One of several exemplary fusion partners includes the ECD of human FZD4, shown in bold in SEQ ID NO: 6 below. According to this exemplary aspect the disclosure provides a method to display a conformationally intact fragment of human FZD4 on the surface of a poxvirus EEV comprising infecting host cells permissive for poxvirus infectivity with a recombinant poxvirus encoding a fusion protein comprising amino acids 20 to 370 of SEQ ID NO: 6. In certain 65 aspects the fusion protein can further comprise a signal peptide, e.g., amino acids 1 to 19 of SEQ ID NO: 6.

(Seq ID NO: 6) MGWSCIILFLVATATGAHSFGDEEERRCDPIRISMCQNLGYNVTKMPNLVG

32

HELQTDAELQLTTFTPLIQYGCSSQLQFFLCSVYVPMCTEKINIPIGPCGG MCLSVKRRCEPVLKEFGFAWPESLNCSKFPPQNDHNHMCMEGPGDEEVPLP

 $\textbf{HKTPIQPGEE} \ TSTTNDTDKVDYEEYSTELIVNTDSESTIDIILSGSTHSPE$ $_{10} \ \textit{tsskkpdyidnsncssvfeiatpepitdnvedhtdtvtytsdsintvsass}$

GESTTDETPEPITDKEDHTVTDTVSYTTVSTSSGIVTTKSTTDDADLYDTY

NDNDTVPPTTVGGSTTSISNYKTKDFVEIFGITALIILSAVAIFCITYYTY

NKRSRKYKTENKV.

Single Underline - leader peptide (amino acids 1-19) Bold - human FZD4 extracellular domain (amino acids 20-163) Italics - A56R stalk, transmembrane, and intra-

membrane (amino acids 164 to 370)

Another exemplary fusion partner includes the ECD of human ErbB2 (Her2), shown in bold in SEQ ID NO: 7 below. According to this exemplary aspect the disclosure provides a method to display a conformationally intact fragment of human Her2 on the surface of a poxvirus EEV comprising infecting host cells permissive for poxvirus infectivity with a recombinant poxvirus encoding a fusion protein comprising amino acids 20 to 855 of SEQ ID NO: 7. In certain aspects the fusion protein can further comprise a signal peptide, e.g., amino acids 1 to 19 of SEQ ID NO: 7.

Her2-A56R

(SEO ID NO: 7)

MGWSCIILFLVATATGAHSSTQVCTGTDMKLRLPASPETHLDMLRHLYQGC QVVQGNLELTYLPTNASLSFLQDIQEVQGYVLIAHNQVRQVPLQRLRIVRG TOLFEDNYALAVLDNGDPLNNTTPVTGASPGGLRELOLRSLTEILKGGVLI ORNPOLCYODTILWKDIFHKNNOLALTLIDTNRSRACHPCSPMCKGSRCWG ESSEDCOSLTRTVCAGGCARCKGPLPTDCCHEQCAAGCTGPKHSDCLACLH FNHSGICELHCPALVTYNTDTFESMPNPEGRYTFGASCVTACPYNYLSTDV GSCTLVCPLHNQEVTAEDGTQRCEKCSKPCARVCYGLGMEHLREVRAVTSA protein, any membrane-associated fragment thereof, or any 45 NIQEFAGCKKIFGSLAFLPESFDGDPASNTAPLQPEQLQVFETLEEITGYL YISAWPDSLPDLSVFONLOVIRGRILHNGAYSLTLOGLGISWLGLRSLREL GSGLALIHHNTHLCFVHTVPWDOLFRNPHOALLHTANRPEDECVGEGLACH vided above, e.g., where the IMP is a multi-pass membrane 50 QLCARGHCWGPGPTQCVNCSQFLRGQECVEECRVLQGLPREYVNARHCLPC ${\tt HPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMPIW}$ $\textbf{KFPDEEGACQPCPINCTHSCVDLDDKGCPAEQRASP} \ TSTTNDTDKVDYEEY$

> FVEIFGITALIILSAVAIFCITYYIYNKRSRKYKTENKV. 60 Single Underline - leader peptide (amino acids 1-19) Bold - human ERBB2 (HER2) extracellular domain (amino acids 20-648) Italics - A56R stalk, transmembrane, and intramembrane (amino acids 649 to 855)

Another exemplary fusion partner includes the ECD of human CD100 (Semaphorin 4D), shown in bold in SEQ ID

ITDNVEDHTDTVTYTSDSINTVSASSGESTTDETPEPITDKEDHTVTDTVS

YTTVSTSSGIVTTKSTTDDADLYDTYNDNDTVPPTTVGGSTTSISNYKTKD

NO: 8 below. According to this exemplary aspect the disclosure provides a method to display a conformationally intact fragment of human CD100 on the surface of a poxvirus EEV comprising infecting host cells permissive for poxvirus infectivity with a recombinant poxvirus encoding a 5 fusion protein comprising amino acids 20 to 935 of SEQ ID NO: 8. In certain aspects the fusion protein can further comprise a signal peptide, e.g., amino acids 1 to 19 of SEQ ID NO: 8.

CD100-A56R

(SEO ID NO: 8)

MGWSCIILFLVATATGAHSFAPIPRITWEHREVHLVQFHEPDIYNYSALLL SEDKDTLYIGAREAVFAVNALNISEKOHEVYWKVSEDKKAKCAEKGKSKOT 15 ECLNYIRVLOPLSATSLYVCGTNAFOPACDHLNLTSFKFLGKNEDGKGRCP FDPAHSYTSVMVDGELYSGTSYNFLGSEPIISRNSSHSPLRTEYAIPWLNE psfvfadvirkspdspdgeddrvyffftevsveyefvfrvlipriarvckg 20 DQGGLRTLQKKWTSFLKARLICSRPDSGLVFNVLRDVFVLRSPGLKVPVFY ALFTPOLNNVGLSAVCAYNLSTAEEVFSHGKYMOSTTVEOSHTKWVRYNGP ${\tt VPKPRPGACIDSEARAANYTSSLNLPDKTLQFVKDHPLMDDSVTPIDNRPR}$ $\verb|LIKKDVNYTQIVVDRTQALDGTVYDVMFVSTDRGALHKAISLEHAVHIIEE|$ TQLFQDFEPVQTLLLSSKKGNRFVYAGSNSGVVQAPLAFCGKHGTCEDCVL ${\tt ARDPYCAWSPPTATCVALHQTESPSRGLIQEMSGDASVCPDKSKGSYRQHF}$ FKHGGTAELKCSQKSNLARVFWKFQNGVLKAESPKYGLMGRKNLLIFNLSE GDSGVYQCLSEERVKNKTVFQVVAKHVLEVKVVPKPVVAPTLSVVQTEGSR IATKVLVASTQGSSPPTPAVQATSSGAITLPPKPAPTGTSCEPKIVINTVP QLHSEKTMYLKSSDTSTTNDTDKVDYEEYSTELIVNTDSESTIDIILSGST HSPETSSKKPDYIDNSNCSSVFEIATPEPITDNVEDHTDTVTYTSDSINTV SASSGESTTDETPEPITDKEDHTVTDTVSYTTVSTSSGIVTTKSTTDDADL

YDTYNDNDTVPPTTVGGSTTSISNYKTKDFVEIFGITALIILSAVAIFCIT YYIYNKRSRKYKTENKV.

Single Underline - leader peptide (amino acids 1-19)

Bold - human CD100 extracellular domain (amino acids 20-728)

Italics - A56R stalk, transmembrane, and intramembrane (amino acids 729 to 935)

In certain aspects, the membrane-associated EEV specific protein fragment includes the transmembrane and intramembrane domains of the vaccinia virus B5R protein, a fragment comprising, consisting of, or consisting essentially of amino acids 276 to 317 of SEQ ID NO: 9. In certain aspects, the membrane-associated EEV specific protein fragment includes the stalk, transmembrane, and intra-membrane domains of the vaccinia virus B5R protein, a fragment comprising, consisting of, or consisting essentially of amino acids 238 to 317 of SEQ ID NO: 9.

SEQ ID NO: 9: WR B5R protein
MKTISVVTLLCVLPAVVYSTCTVPTMNNAKLTSTETSFNDKQKVTFTCDQG
YHSSDPNAVCETDKWKYENPCKKMCTVSDYISELYNKPLYEVNSTMTLSCN
GETKYFRCEEKNGNTSWNDTVTCPNAECQPLQLEHGSCQPVKEKYSFGEYM
TINCDVGYEVIGASYISCTANSWNVIPSCQQKCDMPSLSNGLISGSTFSIG

-continued

GVIHLSCKSGFTLTGSPSSTCIDGKWNPVLPICVRTNEEFDPVDDGPDDET

DLSKLSKDVVQYEQEIESLEATYHIIIVALTIMGVIFLISVIVLVCSCDKN

NDQYKFHKLLP

In certain exemplary aspects the IMP fusion partner for the B5R fragment comprises the extracellular domain of human FZD4, shown in bold in SEQ ID NO: 10 and SEQ ID NO: 11 below. According to this exemplary aspect the disclosure provides a method to display a conformationally intact fragment of human FZD4 on the surface of a poxvirus EEV comprising infecting host cells permissive for poxvirus infectivity with a recombinant poxvirus encoding a fusion protein comprising amino acids 20 to 243 of SEQ ID NO: 10 or amino acids 20 to 281 of SEQ ID NO: 11. In certain aspects the fusion protein can further comprise a signal peptide, e.g., amino acids 1 to 19 of SEQ ID NO: 10.

FZD-B5R (short)

(SEQ ID NO: 10)

MGWSCIILFLVATATGAYAFGDEEERRCDPIRISMCQNLGYNVTKMPNLVG

 ${\tt HELQTDAELQLTTFTPLIQYGCSSQLQFFLCSVYVPMCTEKINIPIGPCGG}$

MCLSVKRRCEPVLKEFGFAWPESLNCSKFPPQNDHNHMCMEGPGDEEVPLP

HKTPIQPGEECHSVGTNSDQYIWVKRSLNCVLKCGYDAGLYSRSAKEC A TY

HIIIVALTIMGVIFLISVIVLVCSCDKNNDQYKFHKLLP.
Single Underline - leader peptide (amino acids 119)
Bold - human FZD4 extracellular domain (amino acids 20-200)

Italics - B5R TM and cytoplasmic tail (amino acids 201-243)

35 FZD-B5R (long)

60

(SEQ ID NO: 11)

 $\underline{\texttt{MGWSCIILFLVATATGAYA}} \textbf{FGDEEERRCDPIRISMCQNLGYNVTKMPNLVG}$

 ${\tt HELQTDAELQLTTFTPLIQYGCSSQLQFFLCSVYVPMCTEKINIPIGPCGG}$

 $_{40}$ MCLSVKRRCEPVLKEFGFAWPESLNCSKFPPQNDHNHMCMEGPGDEEVPLP

HKTPIQPGEECHSVGTNSDQYIWVKRSLNCVLKCGYDAGLYSRSAKE YVRT

NEEFDPVDDGPDDETDLSKLSKDVVQYEQEIESLEATYHIIIVALTIMGVI

45 FLISVIVLVCSCDKNNDQYKEHKLLP.

Single Underline - leader peptide (amino acids 1-19)

 $\ensuremath{\text{Bold}}$ - human FZD4 extracellular domain (amino acids 20-200)

Italics - B5R stalk, TM and cytoplasmic tail (amino acids 201-281)

The disclosure further provides a fusion protein comprising: amino acids 20 to 892 of SEQ ID NO: 2; SEQ ID NO: 3; SEQ ID NO: 4; amino acids 20 to 370 of SEQ ID NO: 6; amino acids 20 to 855 of SEQ ID NO: 7; amino acids 20 to 935 of SEQ ID NO: 8; amino acids 20 to 243 of SEQ ID NO: 10; or amino acids 20 to 281 of SEQ ID NO: 11, any combination thereof, any fragment thereof, or any variant thereof, where the fusion protein, when expressed by a recombinant poxvirus, appears on the surface of a poxvirus extracellular enveloped virion (EEV) in a native conformation.

A recombinant poxvirus EEV comprising any EEV fusion protein as provided herein is also provided.

Method of Selecting Antibodies

This disclosure further provides a method to select binding molecules, e.g., antibodies, antigen-binding antibody

fragments, or antibody like binding molecules that bind to a multi-pass membrane protein interest. The method comprises attaching a recombinant EEV as provided herein to a solid support, where the recombinant EEV can display a multi-pass protein on its surface; providing a display library, 5 e.g., an antibody display library, where the library comprises display packages displaying a plurality of antigen binding domains; contacting the display library with the EEV such that display packages displaying antigen binding domains that specifically binds to the IMP expressed on the EEV can 10 bind thereto; removing unbound display packages; and recovering display packages that display an antigen binding domain specific for the IMP expressed on the EEV.

Any display library comprising a plurality of binding domains, e.g., antibodies, antibody like molecules or other 15 binding molecules is suitable for this method. For example the display library can be a phage display library, a yeast display library or a library constructed in a vaccinia virus vector as described elsewhere herein.

vated prior to attachment to the solid support. For example, the EEV can be inactivated by incubation with Psoralen (Trioxsalen, 4'-aminomethyl-, hydrochloride) in the presence of UV irradiation.

Any suitable solid support can be used. As used herein, a 25 "solid support" is any support capable of binding an EEV, which can be in any of various forms, as is known in the art. Well-known supports include tissue culture plastic, glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, 30 gabbros, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of this disclosure. The support material can have virtually any structural configuration as long as the coupled EEV is capable of binding to a displayed binding molecule such as 35 an antibody. Thus, the support configuration can be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface can be flat such as a sheet, test strip, etc. Typical supports include beads, e.g., magnetic polystyrene beads 40 such as DYNABEADS® that can be pulled out of suspension by a magnet. The support configuration can include a tube, bead, microbead, well, plate, tissue culture plate, petri plate, microplate, microtiter plate, flask, stick, strip, vial, paddle, etc., etc. A solid support can be magnetic or non- 45 magnetic. Those skilled in the art will know many other suitable carriers for binding EEV as provided herein, or will be able to readily ascertain the same. In certain aspects, EEV as provided herein can be attached to the solid support via reaction with, e.g., tosyl groups, epoxy groups, carboxylic 50 acid groups, or amino groups attached to the surface. For example, EEV can be attached to the surface of tosylactivated magnetic beads, e.g., MYONE™ tosylactivated beads. Alternatively, the EEV can be biotinylated and attached to a streptavidin solid surface, e.g., streptavidin 55 coated magnetic beads.

This disclosure employs, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. 60 illustration and not by way of limitation. Such techniques are explained fully in the literature. (See, for example, Sambrook et al., ed. (1989) Molecular Cloning A Laboratory Manual (2nd ed.; Cold Spring Harbor Laboratory Press); Sambrook et al., ed. (1992) Molecular Cloning: A Laboratory Manual, (Cold Springs Harbor Laboratory, NY); D. N. Glover ed., (1985) DNA Cloning, Volumes I and II; Gait, ed. (1984) Oligonucleotide Synthesis; Mullis

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et al. U.S. Pat. No. 4,683,195; Hames and Higgins, eds. (1984) Nucleic Acid Hybridization; Hames and Higgins, eds. (1984) Transcription And Translation: Freshney (1987) Culture Of Animal Cells (Alan R. Liss, Inc.); Immobilized Cells And Enzymes (IRL Press) (1986); Perbal (1984) A Practical Guide To Molecular Cloning; the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Miller and Calos eds. (1987) Gene Transfer Vectors For Mammalian Cells, (Cold Spring Harbor Laboratory); Wu et al., eds., Methods In Enzymology, Vols. 154 and 155; Mayer and Walker, eds. (1987) Immunochemical Methods In Cell And Molecular Biology (Academic Press, London); Weir and Blackwell, eds., (1986) Handbook Of Experimental Immunology, Volumes I-IV; Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1986); and in Ausubel et al. (1989) Current Protocols in Molecular Biology (John Wiley and Sons, Baltimore, Md.).

General principles of antibody engineering are set forth in In certain aspects, the recombinant EEV can be inacti- 20 Borrebaeck, ed. (1995) Antibody Engineering (2nd ed.; Oxford Univ. Press). General principles of protein engineering are set forth in Rickwood et al., eds. (1995) Protein Engineering, A Practical Approach (IRL Press at Oxford Univ. Press, Oxford, Eng.). General principles of antibodies and antibody-hapten binding are set forth in: Nisonoff (1984) Molecular Immunology (2nd ed.; Sinauer Associates, Sunderland, Mass.); and Steward (1984) Antibodies, Their Structure and Function (Chapman and Hall, New York, N.Y.). Additionally, standard methods in immunology known in the art and not specifically described can be followed as in Current Protocols in Immunology, John Wiley & Sons, New York; Stites et al., eds. (1994) Basic and Clinical Immunology (8th ed; Appleton & Lange, Norwalk, Conn.) and Mishell and Shiigi (eds) (1980) Selected Methods in Cellular Immunology (W.H. Freeman and Co., NY).

> Standard reference works setting forth general principles of immunology include Current Protocols in Immunology, John Wiley & Sons, New York; Klein (1982) J., Immunology: The Science of Self-Nonself Discrimination (John Wiley & Sons, NY); Kennett et al., eds. (1980) Monoclonal Antibodies, Hybridoma: A New Dimension in Biological Analyses (Plenum Press, NY); Campbell (1984) "Monoclonal Antibody Technology" in Laboratory Techniques in Biochemistry and Molecular Biology, ed. Burden et al., (Elsevier, Amsterdam); Goldsby et al., eds. (2000) Kuby Immunology (4th ed.; H. Freeman & Co.); Roitt et al. (2001) Immunology (6th ed.; London: Mosby); Abbas et al. (2005) Cellular and Molecular Immunology (5th ed.; Elsevier Health Sciences Division); Kontermann and Dubel (2001) Antibody Engineering (Springer Verlag); Sambrook and Russell (2001) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Press); Lewin (2003) Genes VIII (Prentice Hall, 2003); Harlow and Lane (1988) Antibodies: A Laboratory Manual (Cold Spring Harbor Press); Dieffenbach and Dveksler (2003) PCR Primer (Cold Spring Harbor Press).

All of the references cited above, as well as all references cited herein, are incorporated herein by reference in their entireties. The following examples are offered by way of

EXAMPLES

Example 1: Fusion Protein Construction

IMPs were incorporated into vaccinia virus EEVs using the EEV-specific proteins F13L, A56R, and B5R, by the

following methods. Generally, the extracellular domains of HER2, CD100 (semaphorin 4D), and FZD4 were incorporated as fusions with the single-pass EEV-specific membrane proteins A56R and B5R as diagrammed in FIG. 1A. The mature FZD4-ECD-A56R fusion protein comprises amino acids 20 to 370 of SEQ ID NO: 6, the mature HER2-ECD-A56R fusion protein comprises amino acids 20 to 855 of SEQ ID NO: 7, and the mature CD100-ECD-A56R fusion protein comprises amino acids 20 to 935 SEQ ID NO: 8. FIG. 1B and FIG. 1C show diagrammatically how the multi-pass proteins such as GPCRs and CD20 can be incorporated into EEVs as multi-pass membrane proteins as a fusion with the EEV membrane-associated protein F13L.

Preparation of F13L Fusion Proteins (FZD4-F13L, CD20-F13L, and CXCR4-F13L)

cDNAs encoding the IMPs were cloned in-frame to the vaccinia virus F13L gene encoding the palmitoylated EEV membrane glycoprotein (SEQ ID NO: 1) into the pJEM1 plasmid previously described for the purpose of introduction into vaccinia virus. pJEM1 is a derivative of p7.5/tk described in U.S. Patent Appl. Publ. No. 2013/0288927, and when digested with NcoI or BssHII and BsiWI, contains flanking regions capable of homologous recombination with the vaccinia virus TK gene and the vaccinia virus H5 promoter.

The open reading frame of human membrane protein MS4A1 gene (CD20)(NM_021950.3) was cloned in frame with the vaccinia virus F13L using SOE (Splicing by Overlap Extension) PCR as per standard protocols whereby restriction endonuclease sites NcoI and BsiWI were added to the PCR product by encoding them into the 5' and 3'-most flanking primers respectively. This strategy avoids the introduction of a leader peptide. The final PCR product and pJEM1 were digested with NcoI and BsiWI and the two species were joined via ligation according to standard protocols. The circularized plasmid was then introduced into competent *E. coli* via chemical transformation and colonies were selected on ampicillin-containing agar plates.

MS4A15

tataCCATGgCAACACCCCAGAAATTCAGTAAATG

MS4A1AS

GGTACCGATGCAAATGGCCACATAGGAGAGCTGTCATTTTCTATTGG

F135

(SEQ ID NO: 13)

CCAATAGAAAATGACAGCTCTCCTATGTGGCCATTTGCATCGGTACC

F13AS

(SEQ ID NO: 15)

tataCGTACGTTAATGGTGATGGTGATGATTTTTAACGATTTACTGT

The resulting CD20-F13L fusion protein encoded by the polynucleotide comprises the amino acid sequence SEQ ID NO: 4

The open reading frame of human membrane protein FZD4 (NM_012193.3) was cloned in frame with the vaccinia virus F13L using SOE (Splicing by Overlap Extension) PCR as per standard protocols whereby restriction endonuclease sites BssHII and BsiWI were added to the PCR 65 product by encoding them into the 5' and 3'-most flanking primers, respectively. This strategy provides for the use of

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the leader peptide contained within pJEM1. The final PCR product and pJEM1 were digested with BssHII and BsiWI and the two species were joined via ligation according to the standard protocols. The circularized plasmid was then introduced into competent *E. coli* via chemical transformation and colonies were selected on ampicillin-containing agar plates. PCR primers were specific for FZD4 and F13L and conform to the same general strategy as described for MS4A1. The resulting mature FZD4-F13L fusion protein encoded by the polynucleotide comprises amino acids 20-892 of SEQ ID NO: 2.

The open reading frame of human membrane protein CXCR4 (NM_001008540.1) was cloned in frame with the vaccinia virus F13L using SOE (Splicing by Overlap Exten-15 sion) PCR as per standard protocols whereby restriction endonuclease sites NcoI and BsiWI were added to the PCR product by encoding them into the 5' and 3'-most flanking primers respectively. This strategy avoids the introduction of a leader peptide. The final PCR product and pJEM1 were digested with NcoI and BsiWI and the two species were joined via ligation according to the standard protocol. The circularized plasmid was then introduced into competent E. coli via chemical transformation and colonies were selected on ampicillin-containing agar plates. PCR primers were specific for CXCR4 and F13L and conform to the same general strategy as described for MS4A1. The resulting CXCR4-F13L fusion protein encoded by the polynucleotide comprises the amino acid sequence SEQ ID NO: 3.

The plasmids produced as described above, as well as similar plasmids encoding non-fused ("untagged") versions of CD20, FZD4, CXCR4, CD100, and HER2, were linearized and introduced into vaccinia virus via tri-molecular recombination.

Example 2: Expression of CD20-F13L Fusion Protein on EEV

BHK cells were infected with either IMV encoding the CD20-F13L fusion protein (SEQ ID NO: 4) or Control Western Reserve (WR) virus at a multiplicity of infection (MOI) of 1 virus per cell for two days after which the supernatant containing EEV was harvested and debris removed by low speed centrifugation. Protein G DYNA-BEADS® (110 μL) were pulled down with a magnet and 1 45 mL of PBS+20 μg of purified anti-CD20 antibody was added to the beads. The solution was incubated at room temperature with gentle rotation for 30-60 minutes to allow the antibody to couple to the Protein G beads. Ten µg of purified mIgG1 isotype control was added to the solution to ensure complete blocking, and the solution was incubated at room temperature with gentle rotation for 10-30 additional minutes. Beads were pulled down with the magnet, washed once with 1 mL of PBS and resuspended in 110 μL of PBS.

Fifty μL of Anti-CD20-Pro G DYNABEADS® was added to 1 mL of CD20-F13L or WR EEV supernatant and was incubated at room temperature with gentle rotation for 1 hour. Beads were pelleted using the magnet and unbound supernatant removed. The beads were then washed five times with 1 mL of Dulbecco's Modified Eagle Medium (DMEM) media supplemented with 10% FBS and 1 mM HEPES (10% DMEM). All washes were pooled with the unbound supernatant ("Unbound"). The beads ("Bound") were then resuspended in 1 mL of 10% DMEM. "Unbound" and "Bound" were titered on BSC-1 cells and overlaid with growth medium containing methylcellulose. Plaques were allowed to form for two days and then the cells were fixed and stained with 0.1% Crystal Violet solution. Plaques were

counted to determine the number of plaque forming units (pfu) in the "Unbound" and "Bound" from which the % of EEV bound to the beads could be calculated. Results are shown on Table 2.

TABLE 2

CD20-F13L EEV Binding		
EEV Supernatant	% Bound	
Western Reserve CD20-F13L	10.8% 50.5%	

The % EEV bound to the anti-CD20 coated beads was significantly higher for CD20-F13L EEV fusion protein than it is for the Western Reserve indicating that CD20 is being expressed on the EEV membrane surface.

Example 3: Fusion of CD20 to F13L is More Efficiently Expressed on the EEV Membrane

BHK cells were infected with either IMV encoding the CD20-F13L fusion protein (SEQ ID NO: 4), CD20-A56R fusion protein (SEQ ID NO: 16), CD20 untagged (unfused) or control HER2-A56R Extracellular Domain (ECD) (SEQ ID NO: 7) virus at a MOI=1 for two days after which the supernatant containing EEV was harvested and debris removed by low speed centrifugation. Streptavidin DYNA-BEADS® (200 μ L) were pulled down with a magnet and 0.2 mL of PBS+20 μ g of purified Biotinylated anti-CD20 antibody or Biotinylated anti-HER2 was added to the beads. The solution was incubated at room temperature with gentle rotation for 30 minutes to allow the antibody to couple to the Streptavidin beads. Beads were pulled down with the magnet, washed once with 1 mL of PBS and resuspended in 200 35 μ L of PBS.

CD20-A56R:

(SEQ ID NO: 16) MGWSCHLFLVATATGAHTELIVNTDSESTIDIILSGSTHSPETSSKKPDYI

DNSNCSSVFEIATPEPITDNVEDHTDTVTYTSDSINTVSASSGESTTDETP

EPITDKEDHTVTDTVSYTTVSTSSGIVTTKSTTDDADLYDTYNDNDTVPPT

TVGGSTTSISNYKTKDFVEIFGITALIILSAVAIFCITYYIYNKRSRKYKT

ENKV*MTTPRNSVNG*TFPAEPMKGPIAMQSGPKPLFRRMSSLVGPTQSFFMR

ESKTLGAVQIMNGLFHIALGGLLMIPAGIVAPICVTVWYPLWGGIMYIISG

SLLAATEKNSRKCLVKGKMIMNSLSLFAAISGMILSIMDILNIKISHFLKM

ESLNFIRAHTPYINIYNCEPANPSEKNSPSTQYCYSIQSLFLGILSVMLIF

AFFQELVIAGIVENEWKRTCSRPKSNIVLLSAEEKKEQTIEIKEEVVGLTE

TSSQPKNEEDIEIIPIQEEEEEETETNFPEPPQDQESSPIENDSSP Single Underline - Signal sequence (amino acids 1-

Italics - Truncated A56R (amino acids 20-190) Bold - CD20 Sequence (amino acids 191-506)

Fifty μL of prepared streptavidin beads were added to 1 60 mL of each EEV supernatant and allowed to rotate at room temperature for 45 minutes. Beads were pelleted using the magnet and unbound supernatant removed. The beads were then washed five times with 1 mL of DMEM media supplemented with 10% FBS and 1 mM HEPES (10% DMEM). 65 All washes were pooled with the unbound supernatant ("Unbound"). The beads ("Bound") were then resuspended

in 1 mL of 10% DMEM. "Unbound" and "Bound" were titered on BSC-1 cells and overlaid with growth medium containing methylcellulose. Plaques were allowed to form for two days and then the cells were fixed and stained with 0.1% Crystal Violet solution. Plaques were counted to determine the number of plaque forming units (pfu) in the "Unbound" and "Bound" from which the % of EEV bound to the beads could be calculated. Results are shown in FIG. 2.

The % EEV bound to the anti-CD20 coated beads for CD20-F13L was greater than the % EEV bound for untagged (unfused) or A56R-fused CD20 indicating higher expression of CD20-F13L on the EEV membrane. The lack of binding to the anti-HER2 coated beads confirmed specificity of the assay.

The experiment above was repeated using CD20-F13L fusion protein (SEQ ID NO: 4), CD20 untagged (unfused), FZD-F13L fusion protein (SEQ ID NO: 2), and FZD untagged (unfused). Virus was pulled down using anti-CD20 or anti-FZD coated beads as described above. The data in FIG. 3A (anti-CD20-coated beads) and FIG. 3B (anti-FZD-coated beads) shows that F13L fusion proteins were specifically pulled down by their respective antibodies and were more efficiently incorporated into vaccinia virus than untagged (unfused) proteins.

Example 4: Vaccinia Virus can be engineered to Express Various Antigen-EEV Constructs

BHK cells were infected at a MOI=1 with virus expressing the following antigen constructs: CD20-F13L (SEQ ID NO: 4), CXCR4-F13L (SEQ ID NO: 3), HER2-ECD-A56R (SEQ ID NO: 7), and CD100-ECD-A56R (SEQ ID NO: 8). After two days, the supernatant containing EEV was harvested and debris removed by low speed centrifugation. Streptavidin DYNABEADS® were pulled down with a magnet and for each sample, 50 µL of beads were resuspended in 0.1 mL of PBS+5 µg of purified Biotinylated anti-CD20 antibody, Biotinylated anti-CXCR4 (12G5), Biotinylated anti-CD100 (2503), or Biotinylated anti-HER2. The solutions were incubated at room temperature with gentle rotation for 30 minutes to allow the antibody to couple to the Streptavidin beads. Beads were pulled down with the magnet, washed once with 1 mL of PBS and resuspended in 100 uL of PBS per sample.

One hundred µL of prepared streptavidin beads were added to 1 mL of each EEV supernatant and allowed to rotate at room temperature for 45 minutes. Beads were pelleted using the magnet and unbound supernatant removed. The beads were then washed five times with 1 mL of DMEM media supplemented with 10% FBS and 1 mM HEPES (10% DMEM). All washes were pooled with the 55 unbound supernatant ("Unbound"). The beads ("Bound") were then resuspended in 1 mL of 10% DMEM. "Unbound" and "Bound" were titered on BSC-1 cells and overlaid with growth medium containing methylcellulose. Plaques were allowed to form for two days and then the cells were fixed and stained with 0.1% Crystal Violet solution. Plaques were counted to determine the number of plaque forming units (pfu) in the "Unbound" and "Bound" from which the % of EEV bound to the beads could be calculated. Results are shown in FIG. 4.

All of the antigen-EEV bound specifically to their corresponding antibody-coupled beads indicating efficient expression of the antigen on the EEV membrane.

Example 5: Antigen-EEV can be Directly Coupled to Magnetic Beads for Antibody Selection

BHK cells $(2\times10^8 \text{ cells})$ were infected at a MOI=1 with virus expressing HER2-ECD-A56R (SEQ ID NO: 7), FZD-5 F13L (SEQ ID NO: 2), CXCR4-F13L (SEQ ID NO: 3) or CD100 (semaphorin 4D)-ECD-A56R (SEO ID NO: 8) in one cellSTACK cell culture chamber each (Corning). After two days, the supernatant containing EEV was harvested and debris removed by low speed centrifugation. The clarified supernatant was then spun at 13,000 rpm (28,000×g) for 1 hour to pellet the antigen-EEV. The supernatant was aspirated and the pellet resuspended in 1.5 mL of 1× PBS. The various viruses were transferred to fresh tubes and Psoralen 15 (Trioxsalen, 4'-aminomethyl-, hydrochloride; Sigma) was added to 20 µg/ml final concentration. The EEV and Psoralen were incubated at room temperature for 10 minutes before being irradiated in the STRATALINKER® UV Crosslinker (Stratagene) for 99,999 microjoules. The Pso- 20 ralen/UV procedure ensures that the antigen-EEV is inactivated and therefore unable to form plaques or multiply in any downstream testing.

Tosylactivated MyOne DYNABEADS® (100 µL) were pulled down with a magnet and washed with 1 mL of PBS. 25 The beads were pulled down with the magnet, the PBS removed and 1 mL of each Psoralen/UV inactivated antigen-EEV was added to a separate aliquot of beads. The beads and antigen-EEV were allowed to rotate at 37° C. for 16-20 hours. The beads were pelleted and the supernatant was 30 removed. The beads were blocked with 1 mL of 1×PBS, 10% FBS and 0.5% BSA at 37° C. for 1 hour. The beads were pelleted and washed with 1 mL 1×PBS before being resuspended in 200 µL of 1×PBS.

One hundred microliters of antigen-EEV-coupled beads 35 was added to 1 mL of each respective antibody-EEV supernatant expressing anti-FZD4, anti-CXCR4, anti-CD100, or anti-HER2, as well as to control antibody-EEV. Antibody EEV were produced by infecting BHK cells at a MOI=1 each for 2 days with vaccinia virus encoding both the heavy 40 and light chains of the respective antibodies, and harvesting the supernatants followed by a low speed spin to remove any cells. The virus coupled beads and antibody EEV were allowed to rotate at room temperature for 2 hours. Beads were pelleted using the magnet and unbound supernatants 45 removed. The beads were then washed five times with 1 mL of DMEM media supplemented with 10% FBS and 1 mM HEPES (10% DMEM). All washes were pooled with the unbound supernatant ("Unbound"). The beads ("Bound") were then resuspended in 1 mL of 10% DMEM. "Unbound" 50 resuspended in 150 μ L of 1× PBS. and "Bound" were titered on BSC-1 cells and overlaid with growth medium containing methylcellulose. Plaques were allowed to form for two days and then the cells were fixed and stained with 0.1% Crystal Violet solution. Plaques were counted to determine the number of plaque forming units 55 (pfu) in the "Unbound" and "Bound" from which the % of EEV bound to the beads could be calculated. A diagram of the method is shown in FIG. 5, and results are shown in FIG. 6A (HER2), FIG. 6B (FZD4), FIG. 6C (CXCR4), and FIG. 6D (CD100 ("Sema")).

Antibody-EEV expressing Anti-HER2 was specifically pulled down by beads coupled with HER2-ECD-A56R antigen EEV, Antibody-EEV expressing Anti-FZD was specifically pulled down by beads coupled with FZD-F13L antigen EEV, Antibody-EEV expressing Anti-CXCR4 was 65 specifically pulled down by beads coupled with CXCR4-F13L antigen EEV, and Antibody-EEV expressing Anti42

SEMA was specifically pulled down by beads coupled with Sema-ECD-A56R antigen EEV.

Example 6: Antibody Library Screening

BHK cells were infected at a MOI=1 each with an antibody library (H-IgG-A56R) and L48 (derivative of germline VK1-39) in four cellSTACK cell culture chamber (Corning) $(2\times10^8 \text{ cells per stacker})$. The antibody library contained a diverse population of heavy chain variable domains in full length IgG format, fused in frame to A56R (see US Patent Appl. Publication No. 2013-0288927, which is incorporated herein by reference in its entirety). The diversity of this library was approximately 400 million independent clones. After two days, the supernatant containing EEV was harvested and debris removed by low speed centrifugation. The clarified supernatant was then spun at 13,000 rpm (28,000×g) for 1 hour to pellet the antibody-EEV. The antibody-EEV was resuspended in 1 ml EMEM with 10% FBS and stored at 4 degrees until ready for use. In order to make the antigen virus for panning, BHK cells $(2\times10^8 \text{ cells})$ were infected at a MOI=1.5 with virus expressing FZD4-ECD-A56R (SEQ ID NO: 6) in two cellSTACK cell culture chamber (Corning). After two days, the supernatant containing EEV was harvested and debris removed by low speed centrifugation. The clarified supernatant was then spun at 13,000 rpm (28,000×g) for 1 hour to pellet the antigen-EEV. The supernatant was aspirated and the pellet resuspended in 1.0 mL of 1×PBS. The one mL of the FZD4-ECD-A56R EEV was transferred to a fresh tube and Psoralen (Trioxsalen, 4'-aminomethyl-, hydrochloride; Sigma) was added to 40 μg/ml final concentration. The EEV and Psoralen were incubated at room temperature for 10 minutes before being irradiated in the STRATALINKER® UV Crosslinker (Stratagene) for 99,999 microjoules. The Psoralen/UV procedure ensures that the antigen-EEV is inactivated and therefore unable to form plaques or multiply in any downstream testing.

Tosylactivated MyOne DYNABEADS® (150 µL) were pulled down with a magnet and washed with 1 mL of PBS, two times. The beads were pulled down with the magnet, the PBS removed and the 1 mL of Psoralen/UV inactivated FZD4-ECD-A56R was added to the beads. The beads and antigen-EEV were allowed to rotate at 37° C. for 18-20 hours. The beads were pelleted and the supernatant was removed. The beads were blocked with 1 mL of 1× PBS. 10% FBS and 0.5% BSA at 37° C. for 2 hours. The beads were pelleted and washed with 1 mL 1×PBS before being

Fifty microliters of FZD4-ECD-A56R-coupled beads were added to 1 mL of the antibody-EEV library. The FZD4-ECD-A56R coupled beads and antibody EEV were allowed to rotate at room temperature for 2 hours. Beads were pelleted using the magnet and unbound supernatant removed. The beads were then washed five times with 1 mL of DMEM media supplemented with 10% FBS and 1 mM HEPES (10% DMEM). All washes were pooled with the unbound supernatant ("Unbound"). The beads ("Bound") were then resuspended in 1 mL of 10% DMEM. "Unbound" and "Bound" were titered on BSC-1 cells and overlaid with growth medium containing methylcellulose. Plaques were allowed to form for two days and then the cells were fixed and stained with 0.1% Crystal Violet solution. The remaining bound virus (990 µl) was divided among 5 T175 flasks containing confluent BSC1 cells and allowed to amplify in DMEM 2.5% containing 1 mg/ml G418 for 3 days. The cells

were then harvested, and the virus released by three cycles of freeze/thaw, and the virus titered.

For the second round of selection (Rd2), the amplified Heavy chains from round 1 were co-infected along with fresh L48 into one cellSTACK of BHK. Antibody EEV was 5 harvested as described above. For each round of panning, fresh FZD-ECD-A56R antigen virus was produced, concentrated, inactivated and coupled to beads as described above. Fifty microliters of FZD4-ECD-A56R coupled beads was added to 1 mL antibody-EEV Rd2. The FZD4-ECD-A56R coupled beads and antibody EEV were allowed to rotate at room temperature for 2 hours. Beads were pelleted using the magnet and unbound supernatant removed. The beads were then washed five times with 1 mL of DMEM media supplemented with 10% FBS and 1 mM HEPES (10% DMEM). All washes were pooled with the unbound supernatant ("Unbound"). The beads ("Bound") were then resuspended in 1 mL of 10% DMEM. "Unbound" and "Bound" were containing methylcellulose. Plaques were allowed to form for two days and then the cells were fixed and stained with 0.1% Crystal Violet solution. The remaining bound virus (990 ul) was divided among 5 T175 flasks containing confluent BSC1 cells and allowed to amplify in DMEM 25 2.5% containing 1 mg/ml G418 for 3 days. The cells were then harvested, and the virus released by three cycles of freeze/thaw, and the virus tittered.

Three additional cycles of panning (Rd3, Rd4, and Rd5) were performed as described above.

Rounds 3, 4 and 5 were tested for enrichment by infected A431 cells in 6 well plate at a MOI=1 with each amplified VH round and L48. After an overnight infection the cells were harvested and split in half. One half was stained with 10 μg/ml FZD-His, followed by anti-His-Dyelight650 and anti-Fab-FITC. The other half was stained with 10 µg/ml CD100-His (negative control), followed by anti-His-Dyelight650 and anti-Fab-FITC. The data shown in FIG. 7 shows increasing enrichment per round of selection. Anti- 40 bodies from round 5 were sub-cloned into a mammalian expression vector to be expressed as full length soluble IgG and transfected (along with L48 in a mammalian expression vector). The resulting antibodies present in the supernatant were tested by flow cytometry for binding to FZD4 trans- 45 fected CHO cells and the absence of binding to CXCR4 transfected CHO cells. A number of antibodies that bound specifically to FZD were identified.

Example 7: Dual Tag/Antigen EEV can be Coupled to Magnetic Beads

BHK cells were infected at two virions per cell where one virion was Hemagglutinin tag (HA)-A56R (SEQ ID NO: 17) and the second was FZD4-F13L (SEQ ID NO: 2) in order to 55 yield EEV expressing both the HA tag and FZD4 antigen on its surface or infected at one virion per cell with each individual virus. After two days, the supernatant containing EEV was harvested and debris removed by low speed centrifugation. Protein G magnetic beads (150 µL) were 60 pulled down with a magnet and 1 mL of PBS+30 µg of purified anti-FZD4 antibody (C6073) was added to the beads. The solution was incubated at room temperature with gentle rotation for 25 minutes to allow the antibody to couple to the Protein G beads. Beads were pulled down with 65 the magnet, washed once with 1 mL of PBS and resuspended in 300 µL of DMEM+10% FBS. Anti-HA-tag magnetic

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beads (ThermoFisher, 150 µl) pulled down with a magnet and washed once with 1 mL of PBS before resuspending in 150 µl of PBS.

HA-A56R

(SEQ ID NO: 17)

MGWSCIILFLVATATGAHSYPYDVPDYATSTTNDTDKVDYEEYSTELIVNT

DSESTIDIILSGSTHSPETSSKKPDYIDNSNCSSVFEIATPEPITDNVEDH

TDTVTYTSDSINTVSASSGESTTDETPEPITDKEDHTVTDTVSYTTVSTSS

GIVTTKSTTDDADLYDTYNDNDTVPPTTVGGSTTSISNYKTKDFVEIFGIT

ALIILSAVAIFCITYYIYNKRSRKYKTENKV Single Underline - Signal sequence (amino acids 1-19) Bold - HA Tag (amino acids 20-29) Italics - Truncated A56R (amino acids 30-235)

Fifty µL of prepared anti-HA-tag beads or 100 µl of titered on BSC-1 cells and overlaid with growth medium 20 prepared anti-FZD4 Protein G were added to 1 mL of each EEV supernatant and allowed to rotate at room temperature for 60 minutes. Beads were pelleted using the magnet and unbound supernatant removed. The beads were then washed five times with 1 mL of DMEM media supplemented with 10% FBS and 1 mM HEPES (10% DMEM). All washes were pooled with the unbound supernatant ("Unbound"). The beads ("Bound") were then resuspended in 1 mL of 10% DMEM. "Unbound" and "Bound" were titered on BSC-1 cells and overlaid with growth medium containing methylcellulose. Plaques were allowed to form for two days and then the cells were fixed and stained with 0.1% Crystal Violet solution. Plaques were counted to determine the number of plaque forming units (pfu) in the "Unbound" and "Bound" from which the % of EEV bound to the beads could be calculated. Results are shown in FIG. 8. EEV expressing both fusion proteins were pulled down by either antibody.

> Example 8: Dual Tag/Antigen EEV can be Coupled to Magnetic Beads and Used to Capture mAb EEV

BHK cells (2×10^8 cells) were infected at two virions per cell where one virion was HA-A56R (SEQ ID NO: 17) and the second was CXCR4-F13L (SEQ ID NO: 3) in order to yield EEV expressing both the HA tag and CXCR4 antigen on its surface. After two days, the supernatant containing EEV was harvested and debris removed by low speed centrifugation. The clarified supernatant was then spun at 13,000 rpm (28,000×g) for 1 hour to pellet the tag/antigen-EEV. The supernatant was aspirated and the pellet resus-50 pended in 1 mL of 1× PBS. Psoralen (Trioxsalen, 4'-aminomethyl-, hydrochloride; Sigma) was added to 40 ug/ml final concentration. The EEV and Psoralen were incubated at room temperature for 10 minutes before being irradiated in the Stratalinker UV Crosslinker (Stratagene) for 99,999 microjoules twice. The Psoralen/UV procedure ensures that the antigen-EEV is inactivated and therefore unable to form plaques or multiply in any downstream testing.

Anti-CXCR4 EEV and anti-HER2 mAb EEV were produced by infecting BHK cells at two virions per cell where one virion was specific heavy chain and the second was specific light chain. After two days, supernatants containing the anti-CXCR4 EEV and the anti-HER2 EEV were harvested and debris removed by low speed centrifugation.

Three hundred microliters of anti-HA magnetic beads were washed with 1 mL of PBS and then resuspended in one milliliter of the Psoralen/UV inactivated HA/CXCR4 EEV. The beads and EEV were incubated at room temperature

with gentle rotation for 90 minutes to allow the EEV to couple with the anti-HA beads. Beads were pulled down with the magnet, washed once with 1 mL of PBS and resuspended in 300 µL of PBS.

One hundred µL of HA/CXCR4 EEV coupled to the anti-HA beads was added to 1 mL of each mAb EEV supernatant and incubated at room temperature with gentle rotation for 1-1.5 hours. Beads were pelleted using the magnet and unbound supernatant removed. The beads were then washed five times with 1 mL of DMEM media supplemented with 10% FBS and 1 mM HEPES (10% DMEM). All washes were pooled with the unbound supernatant ("Unbound"). The beads ("Bound") were then resuspended in 1 mL of 10% DMEM. "Unbound" and "Bound" were titered on BSC-1 cells and overlaid with growth medium 15 containing methylcellulose. Plaques were allowed to form for two days and then the cells were fixed and stained with 0.1% Crystal Violet solution. Plaques were counted to determine the number of plaque forming units (pfu) in the "Unbound" and "Bound" from which the % of EEV bound 20 to the beads could be calculated. Results are shown in FIG. 9. Anti-CXCR4 EEV were specifically captured by the beads coated with EEV co-expressing HA-A45R and CXCR4-F13L.

Example 9: Antigen-EEV can be Biotinylated for Coupling to Magnetic Beads

BHK cells were infected at a MOI=1 with virus expressing FZD4-F13L, FZD4-ECD-A56R or CD20-F13L. After 30 two days, the supernatant containing EEV was harvested and debris removed by low speed centrifugation. The clarified

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supernatant was then spun at 13,000 rpm for 1 hour to pellet the antigen-EEV. The supernatant was aspirated and the pellet resuspended in 1-2 mL of 1×PBS. To biotinylate the EEV, 2.5 μ L of Biotin-XX SSE stock solution in 1×PBS (FLUOREPORTER® Cell Surface Biotinylation Kit, Molecular Probes) was added to the 1 mL of each EEV in PBS and incubated on ice for 30 minutes. Fifty μ L of 1M Tris, pH 8 was added to quench each reaction.

To couple the Biotin-EEV to beads, 150 μL of Streptavidin DYNABEADS® were pelleted and washed once with 1 mL of 1×PBS. The beads were resuspended in 150 μL and 50 μL was added to 1 mL of Eagle's Minimum Essential Medium (EMEM) media containing 10% FBS and 1 mM HEPES (10% EMEM). Fifty μL of Biotin-EEV was added to the beads and media and allowed to rotate at room temperature for 1 hour. Beads were pelleted using the magnet and unbound supernatant removed. The beads were then washed five times with 1 mL of DMEM media supplemented with 10% FBS and 1 mM HEPES (10% DMEM). All washes were pooled with the unbound supernatant ("Unbound"). The beads ("Bound") were then resuspended in 1 mL of 10% DMEM. "Unbound" and "Bound" were titered on BSC-1 cells and overlaid with growth medium containing methylcellulose. Plaques were allowed to form for two days and then the cells were fixed and stained with 0.1% Crystal Violet solution. Plaques were counted to determine the number of plaque forming units (pfu) in the "Unbound" and "Bound" from which the % of EEV bound to the beads could be calculated. Results are shown in FIG.

Antigen-EEV was able to be biotinylated and coupled to magnetic Streptavidin beads.

SEQUENCE LISTING

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<223> OTHER INFORMATION: F13L
<400> SEQUENCE: 1
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Glu Thr Leu Pro Glu Asn Met Asp Phe Arg Ser Asp His Leu Thr Thr 20 25 30
Phe Glu Cys Phe Asn Glu Ile Ile Thr Leu Ala Lys Lys Tyr Ile Tyr 35 \  \  \, 40 \  \  \, 45
Ile Ala Ser Phe Cys Cys Asn Pro Leu Ser Thr Thr Arg Gly Ala Leu
                            55
Ile Phe Asp Lys Leu Lys Glu Ala Ser Glu Lys Gly Ile Lys Ile Ile 65 \phantom{00}70\phantom{00}75\phantom{00}75\phantom{00} 80
Val Leu Leu Asp Glu Arg Gly Lys Arg Asn Leu Gly Glu Leu Gln Ser
His Cys Pro Asp Ile Asn Phe Ile Thr Val Asn Ile Asp Lys Lys Asn
Asn Val Gly Leu Leu Gly Cys Phe Trp Val Ser Asp Asp Glu Arg
                                 120
Cys Tyr Val Gly Asn Ala Ser Phe Thr Gly Gly Ser Ile His Thr Ile
                   135
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Lys Thr Leu Gly Val Tyr Ser Asp Tyr Pro Pro Leu Ala Thr Asp Leu Arg Arg Arg Phe Asp Thr Phe Lys Ala Phe Asn Ser Ala Lys Asn Ser Trp Leu Asn Leu Cys Ser Ala Ala Cys Cys Leu Pro Val Ser Thr Ala Tyr His Ile Lys Asn Pro Ile Gly Gly Val Phe Phe Thr Asp Ser Pro Glu His Leu Leu Gly Tyr Ser Arg Asp Leu Asp Thr Asp Val Val Ile Asp Lys Leu Lys Ser Ala Lys Thr Ser Ile Asp Ile Glu His Leu Ala Ile Val Pro Thr Thr Arg Val Asp Gly Asn Ser Tyr Tyr Trp Pro Asp Ile Tyr Asn Ser Ile Ile Glu Ala Ala Ile Asn Arg Gly Val Lys Ile 265 Arg Leu Leu Val Gly Asn Trp Asp Lys Asn Asp Val Tyr Ser Met Ala 280 Thr Ala Arg Ser Leu Asp Ala Leu Cys Val Gln Asn Asp Leu Ser Val 295 Lys Val Phe Thr Ile Gln Asn Asn Thr Lys Leu Leu Ile Val Asp Asp 310 315 Glu Tyr Val His Ile Thr Ser Ala Asn Phe Asp Gly Thr His Tyr Gln 330 Asn His Gly Phe Val Ser Phe Asn Ser Ile Asp Lys Gln Leu Val Ser 345 Glu Ala Lys Lys Ile Phe Glu Arg Asp Trp Val Ser Ser His Ser Lys 360 Ser Leu Lys Ile 370 <210> SEQ ID NO 2 <211> LENGTH: 892 <212> TYPE: PRT <213> ORGANISM: Artificial Sequence <223> OTHER INFORMATION: FZD (FL) - F13L Fusion Protein <400> SEQUENCE: 2 Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly Ala His Ser Phe Gly Asp Glu Glu Glu Arg Arg Cys Asp Pro Ile Arg Ile Ser Met Cys Gln Asn Leu Gly Tyr Asn Val Thr Lys Met Pro Asn Leu Val Gly His Glu Leu Gln Thr Asp Ala Glu Leu Gln Leu Thr Thr Phe Thr Pro Leu Ile Gln Tyr Gly Cys Ser Ser Gln Leu Gln Phe Phe Leu Cys Ser Val Tyr Val Pro Met Cys Thr Glu Lys Ile Asn Ile Pro Ile Gly Pro Cys Gly Gly Met Cys Leu Ser Val Lys Arg Arg Cys Glu 105 Pro Val Leu Lys Glu Phe Gly Phe Ala Trp Pro Glu Ser Leu Asn Cys 120

Ser	Lys 130	Phe	Pro	Pro	Gln	Asn 135	Asp	His	Asn	His	Met 140	СЛа	Met	Glu	Gly
Pro 145	Gly	Asp	Glu	Glu	Val 150	Pro	Leu	Pro	His	Lys 155	Thr	Pro	Ile	Gln	Pro 160
Gly	Glu	Glu	Cys	His 165	Ser	Val	Gly	Thr	Asn 170	Ser	Asp	Gln	Tyr	Ile 175	Trp
Val	Lys	Arg	Ser 180	Leu	Asn	Cys	Val	Leu 185	Lys	Сув	Gly	Tyr	Asp 190	Ala	Gly
Leu	Tyr	Ser 195	Arg	Ser	Ala	Lys	Glu 200	Phe	Thr	Asp	Ile	Trp 205	Met	Ala	Val
Trp	Ala 210	Ser	Leu	СЛа	Phe	Ile 215	Ser	Thr	Ala	Phe	Thr 220	Val	Leu	Thr	Phe
Leu 225	Ile	Asp	Ser	Ser	Arg 230	Phe	Ser	Tyr	Pro	Glu 235	Arg	Pro	Ile	Ile	Phe 240
Leu	Ser	Met	Cys	Tyr 245	Asn	Ile	Tyr	Ser	Ile 250	Ala	Tyr	Ile	Val	Arg 255	Leu
Thr	Val	Gly	Arg 260	Glu	Arg	Ile	Ser	Сув 265	Asp	Phe	Glu	Glu	Ala 270	Ala	Glu
Pro	Val	Leu 275	Ile	Gln	Glu	Gly	Leu 280	Lys	Asn	Thr	Gly	Сув 285	Ala	Ile	Ile
Phe	Leu 290	Leu	Met	Tyr	Phe	Phe 295	Gly	Met	Ala	Ser	Ser 300	Ile	Trp	Trp	Val
Ile 305	Leu	Thr	Leu	Thr	Trp 310	Phe	Leu	Ala	Ala	Gly 315	Leu	Lys	Trp	Gly	His 320
Glu	Ala	Ile	Glu	Met 325	His	Ser	Ser	Tyr	Phe 330	His	Ile	Ala	Ala	Trp 335	Ala
Ile	Pro	Ala	Val 340	Lys	Thr	Ile	Val	Ile 345	Leu	Ile	Met	Arg	Leu 350	Val	Asp
Ala	Asp	Glu 355	Leu	Thr	Gly	Leu	Сув 360	Tyr	Val	Gly	Asn	Gln 365	Asn	Leu	Asp
Ala	Leu 370	Thr	Gly	Phe	Val	Val 375	Ala	Pro	Leu	Phe	Thr 380	Tyr	Leu	Val	Ile
Gly 385	Thr	Leu	Phe	Ile	Ala 390	Ala	Gly	Leu	Val	Ala 395	Leu	Phe	Lys	Ile	Arg 400
Ser	Asn	Leu	Gln	Lys 405	Asp	Gly	Thr	Lys	Thr 410	Asp	Lys	Leu	Glu	Arg 415	Leu
Met	Val	Lys	Ile 420	Gly	Val	Phe	Ser	Val 425	Leu	Tyr	Thr	Val	Pro 430	Ala	Thr
Cys	Val	Ile 435	Ala	Сув	Tyr	Phe	Tyr 440	Glu	Ile	Ser	Asn	Trp 445	Ala	Leu	Phe
Arg	Tyr 450	Ser	Ala	Asp	Asp	Ser 455	Asn	Met	Ala	Val	Glu 460	Met	Leu	Lys	Ile
Phe 465	Met	Ser	Leu	Leu	Val 470	Gly	Ile	Thr	Ser	Gly 475	Met	Trp	Ile	Trp	Ser 480
Ala	Lys	Thr	Leu	His 485	Thr	Trp	Gln	Lys	Cys 490	Ser	Asn	Arg	Leu	Val 495	Asn
Ser	Gly	Lys	Val 500	Lys	Arg	Glu	Lys	Arg 505	Gly	Asn	Gly	Trp	Val 510	Lys	Pro
Gly	ГЛа	Gly 515	Ser	Glu	Thr	Val	Val 520	Met	Trp	Pro	Phe	Ala 525	Ser	Val	Pro
Ala	Gly 530	Ala	Lys	СЛа	Arg	Leu 535	Val	Glu	Thr	Leu	Pro 540	Glu	Asn	Met	Asp

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Phe	Arg	Glu 35	Glu	Asn	Ala	Asn	Phe 40	Asn	Lys	Ile	Phe	Leu 45	Pro	Thr	Ile
Tyr	Ser 50	Ile	Ile	Phe	Leu	Thr 55	Gly	Ile	Val	Gly	Asn 60	Gly	Leu	Val	Ile
Leu 65	Val	Met	Gly	Tyr	Gln 70	Lys	Lys	Leu	Arg	Ser 75	Met	Thr	Asp	Lys	Tyr 80
Arg	Leu	His	Leu	Ser 85	Val	Ala	Asp	Leu	Leu 90	Phe	Val	Ile	Thr	Leu 95	Pro
Phe	Trp	Ala	Val 100	Asp	Ala	Val	Ala	Asn 105	Trp	Tyr	Phe	Gly	Asn 110	Phe	Leu
CÀa	Lys	Ala 115	Val	His	Val	Ile	Tyr 120	Thr	Val	Asn	Leu	Tyr 125	Ser	Ser	Val
Leu	Ile 130	Leu	Ala	Phe	Ile	Ser 135	Leu	Asp	Arg	Tyr	Leu 140	Ala	Ile	Val	His
Ala 145	Thr	Asn	Ser	Gln	Arg 150	Pro	Arg	Lys	Leu	Leu 155	Ala	Glu	Lys	Val	Val 160
Tyr	Val	Gly	Val	Trp 165	Ile	Pro	Ala	Leu	Leu 170	Leu	Thr	Ile	Pro	Asp 175	Phe
Ile	Phe	Ala	Asn 180	Val	Ser	Glu	Ala	Asp 185	Asp	Arg	Tyr	Ile	Cys 190	Asp	Arg
Phe	Tyr	Pro 195	Asn	Asp	Leu	Trp	Val 200	Val	Val	Phe	Gln	Phe 205	Gln	His	Ile
Met	Val 210	Gly	Leu	Ile	Leu	Pro 215	Gly	Ile	Val	Ile	Leu 220	Ser	CAa	Tyr	Сув
Ile 225	Ile	Ile	Ser	ГЛа	Leu 230	Ser	His	Ser	Lys	Gly 235	His	Gln	ГÀв	Arg	Lys 240
Ala	Leu	Lys	Thr	Thr 245	Val	Ile	Leu	Ile	Leu 250	Ala	Phe	Phe	Ala	Сув 255	Trp
Leu	Pro	Tyr	Tyr 260	Ile	Gly	Ile	Ser	Ile 265	Asp	Ser	Phe	Ile	Leu 270	Leu	Glu
Ile	Ile	Lys 275	Gln	Gly	CAa	Glu	Phe 280	Glu	Asn	Thr	Val	His 285	ГÀЗ	Trp	Ile
Ser	Ile 290	Thr	Glu	Ala	Leu	Ala 295	Phe	Phe	His	Сув	Cys	Leu	Asn	Pro	Ile
Leu 305	Tyr	Ala	Phe		Gly 310			Phe	Lys	Thr 315		Ala	Gln	His	Ala 320
Leu	Thr	Ser	Val	Ser 325	Arg	Gly	Ser	Ser	Leu 330	Lys	Ile	Leu	Ser	Lys 335	Gly
ГÀа	Arg	Gly	Gly 340	His	Ser	Ser	Val	Ser 345	Thr	Glu	Ser	Glu	Ser 350	Ser	Ser
Phe	His	Ser 355	Ser	Met	Trp	Pro	Phe 360	Ala	Ser	Val	Pro	Ala 365	Gly	Ala	Lys
CÀa	Arg 370	Leu	Val	Glu	Thr	Leu 375	Pro	Glu	Asn	Met	Asp 380	Phe	Arg	Ser	Asp
His 385	Leu	Thr	Thr	Phe	Glu 390	СЛа	Phe	Asn	Glu	Ile 395	Ile	Thr	Leu	Ala	Lys 400
ГÀа	Tyr	Ile	Tyr	Ile 405	Ala	Ser	Phe	Cys	Cys 410	Asn	Pro	Leu	Ser	Thr 415	Thr
Arg	Gly	Ala	Leu 420	Ile	Phe	Asp	Lys	Leu 425	Lys	Glu	Ala	Ser	Glu 430	Lys	Gly
Ile	Lys	Ile	Ile	Val	Leu	Leu	Asp	Glu	Arg	Gly	Lys	Arg	Asn	Leu	Gly

435		440	445
Glu Leu Gln Ser	His Cys Pro	Asp Ile Asn F	Phe Ile Thr Val Asn Ile
450	455		460
Asp Lys Lys Asn	Asn Val Gly		Gly Cys Phe Trp Val Ser
465	470		175 480
Asp Asp Glu Arg	Cys Tyr Val	Gly Asn Ala S	Ser Phe Thr Gly Gly Ser
	485	490	495
Ile His Thr Ile		Gly Val Tyr S	Ser Asp Tyr Pro Pro Leu
500		505	510
Ala Thr Asp Leu	Arg Arg Arg	Phe Asp Thr F	Phe Lys Ala Phe Asn Ser
515		520	525
Ala Lys Asn Ser	Trp Leu Asn	Leu Cys Ser A	Ala Ala Cys Cys Leu Pro
530	535		540
Val Ser Thr Ala	Tyr His Ile		Tle Gly Gly Val Phe Phe
545	550		555 560
Thr Asp Ser Pro	Glu His Leu	Leu Gly Tyr S	Ger Arg Asp Leu Asp Thr
	565	570	575
Asp Val Val Ile	Asp Lys Leu	Lys Ser Ala I	Lys Thr Ser Ile Asp Ile
580		585	590
Glu His Leu Ala 595	Ile Val Pro	Thr Thr Arg V	Val Asp Gly Asn Ser Tyr 605
Tyr Trp Pro Asp	Ile Tyr Asn	Ser Ile Ile G	Glu Ala Ala Ile Asn Arg
610	615		620
Gly Val Lys Ile	Arg Leu Leu		Trp Asp Lys Asn Asp Val
625	630		640
Tyr Ser Met Ala	Thr Ala Arg 645	Ser Leu Asp A	Ala Leu Cys Val Gln Asn 655
Asp Leu Ser Val 660	Lys Val Phe	Thr Ile Gln A	Asn Asn Thr Lys Leu Leu 670
Ile Val Asp Asp	Glu Tyr Val	His Ile Thr S	Ser Ala Asn Phe Asp Gly
675		680	685
Thr His Tyr Gln	Asn His Gly	Phe Val Ser F	Phe Asn Ser Ile Asp Lys
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Ser His Ser Lys	Ser Leu Lys 725	Ile	
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20		25	30
Arg Met Ser Ser 35	Leu Val Gly	Pro Thr Gln S	Ger Phe Phe Met Arg Glu 45
Ser Lys Thr Leu	Gly Ala Val	Gln Ile Met A	Asn Gly Leu Phe His Ile
50	55		60
Ala Leu Gly Gly	Leu Leu Met	Ile Pro Ala G	Gly Ile Tyr Ala Pro Ile

Cys Val Thr Val Targ Tyr Pro Leu Try Gly Gly Ite Met Tyr Ite See Gly See Leu Leu Ala Ala Targ Gly Gly Rev Leu Leu Leu Ala Ala Targ Gly See Leu Leu Leu Ala Ala Targ Gly See Leu Leu Leu Leu Leu Ala Ala Targ Gly See Leu Leu Leu Leu Leu Leu Ala Ala Targ Gly See Leu Leu Leu Leu Leu Leu Leu Ala Ala Targ Targ Leu Leu Leu Leu Leu Leu Ala Ala Targ Targ Leu Leu Leu Leu Leu Leu Ala Ala Targ Targ Leu Leu Ala Ala Targ Ta						7.0					7.5					0.0
Ser Gly Ser Leu Leu Ala Ala Thr Glu Lys Asn Ser Arg Lys Cys Leu 100 110 110 110 1115 1115 1115 1115 11	65					70					75					80
Val Lys Gly Lys Met He Met Asn Ser Leu Ser Leu Asn Lys He Ala Ala He 115	CÀa	Val	Thr	Val	_	Tyr	Pro	Leu	Trp	_	Gly	Ile	Met	Tyr		Ile
Ser Gly Met Ile Leu Ser Ile Met Asp Ile Leu Ile Leu	Ser	Gly	Ser		Leu	Ala	Ala	Thr		Lys	Asn	Ser	Arg		Cys	Leu
His Phe Leu Lye Met Glu Ser Leu Aen Phe He Arg Ala His Thr Pro 148 Pro 150 Pro 155 Arg Ala Ala His Thr Pro 150 Pro 150 Arg Ala Ala Ala Arg	Val	Lys		ГÀз	Met	Ile	Met		Ser	Leu	Ser	Leu		Ala	Ala	Ile
145	Ser		Met	Ile	Leu	Ser		Met	Asp	Ile	Leu		Ile	Lys	Ile	Ser
See Pro See Ring Gin Tyr Cys Tyr Ring Fine Gin G		Phe	Leu	Lys	Met		Ser	Leu	Asn	Phe		Arg	Ala	His	Thr	
180	Tyr	Ile	Asn	Ile		Asn	CAa	Glu	Pro		Asn	Pro	Ser	Glu		Asn
195	Ser	Pro	Ser		Gln	Tyr	CAa	Tyr		Ile	Gln	Ser	Leu		Leu	Gly
Ser Asn Ile Val Leu Leu 230 Ser Ala Glu Glu Lys Lys Lys Glu Gln Thr Ile 240 Ser Asn Ile Val Leu Leu 230 Ser Ala Glu Glu Lys Lys Lys Glu Gln Thr Ile 240 Glu Ile Lys Glu Glu Asp Ile Glu Ile Ile Pro Ile Glu Thr Ser Ser Glu Pro 255 Glu	Ile	Leu		Val	Met	Leu	Ile		Ala	Phe	Phe	Gln		Leu	Val	Ile
230	Ala		Ile	Val	Glu	Asn		Trp	Lys	Arg	Thr		Ser	Arg	Pro	Lys
1		Asn	Ile	Val	Leu		Ser	Ala	Glu	Glu		ГÀа	Glu	Gln	Thr	
Second S	Glu	Ile	Lys	Glu		Val	Val	Gly	Leu		Glu	Thr	Ser	Ser		Pro
See Pro 275 See See See Pro Met Try Pro Pro Ala See Vala See Pro Ala See See Pro Met Try Pro Pro Ala See See Pro Met Try Pro Pro Ala See See Pro Ala See See Pro Ala See See Pro Ala See See Pro Ala See Ala Ala	ГÀв	Asn	Glu		Asp	Ile	Glu	Ile		Pro	Ile	Gln	Glu		Glu	Glu
290	Glu	Glu		Glu	Thr	Asn	Phe		Glu	Pro	Pro	Gln		Gln	Glu	Ser
315	Ser		Ile	Glu	Asn	Asp		Ser	Pro	Met	Trp		Phe	Ala	Ser	Val
325		Ala	Gly	Ala	Lys		Arg	Leu	Val	Glu		Leu	Pro	Glu	Asn	
Pro Leu Ser 355 Thr Thr Arg 21 str Ala 26 str Leu 11e Phe Asp 2365 Lys Lys Lys 24 str Lys Glu 360 Ala Ser 370 str Glu Lys Gly 11e Lys 375 Ile Ile Val Leu Leu Leu Asp 380 Asp Glu Arg Gly 370 Arg Asp 375 Ile Val Leu Leu Leu Leu Asp 380 Asp Glu Arg Gly 380 Arg Asp 380 Ile Asp 400 Lys Asp Arg Asp Asp Leu Gly 390 Leu Gly 390 Leu Gly Asp 395 Pro Asp Ile Asp 400 Arg 410 Arg 61y Leu Leu Leu Leu Gly 415 Arg 410 Cys Phe Trp Val Ser Asp Asp Asp Asp 420 Arg Cys Trp Val Gly Asp Asp Asp 425 Arg 425 Trp Val Gly Asp Asp Asp Asp 425 Arg 425 Trp Leu Gly Val Trp Ser 440 Phe Trp 435 Gly Ser Ile His Trp 440 Ile Lys Trp 440 Arg 460 Arg 445 Arg 460 Arg 460 Arg 460 Arg 460 Arg 460 Arg 480	Asp	Phe	Arg	Ser		His	Leu	Thr	Thr		Glu	CAa	Phe	Asn		Ile
355	Ile	Thr	Leu		rys	rys	Tyr	Ile		Ile	Ala	Ser	Phe		CAa	Asn
370 375 380 Lys Arg Asn Leu Gly Glu Leu Gln Ser His Cys Pro Asp Ile Asn Phe 385 710 710 710 710 710 710 710 710 710 710	Pro	Leu		Thr	Thr	Arg	Gly		Leu	Ile	Phe	Asp		Leu	ГÀЗ	Glu
385 390 395 400 Ile Thr Val Asn Ile Asp Lys Lys Asn Asn Val Gly Leu Leu Leu Gly 415 Cys Phe Trp Val Ser Asp Asp Asp Glu Arg Cys Tyr Val Gly Asn Ala Ser 420 Phe Thr Gly Gly Ser Ile His Thr 140 1le Lys Thr Leu Gly Val Tyr Ser 435 Asp Tyr Pro Pro Leu Ala Thr Asp Leu Arg Arg Arg Arg Phe Asp Thr Phe 450 Lys Ala Phe Asn Ser Ala Lys Asn Ser Trp Leu Asn Leu Cys Ser Ala 465 Ala Cys Cys Leu Pro Val Ser Thr Ala Tyr His Ile Lys Asn Pro Ile	Ala		Glu	Lys	Gly	Ile	-	Ile	Ile	Val	Leu		Asp	Glu	Arg	Gly
Cys Phe Trp Val Ser Asp Asp Asp Asp Asp Asp Asp Ala Ser Asp		Arg	Asn	Leu	Gly		Leu	Gln	Ser	His		Pro	Asp	Ile	Asn	
Phe Thr Gly Gly Ser Ile His Thr Ile Lys Thr Leu Gly Val Tyr Ser 435 Asp Tyr Pro Pro Leu Ala Thr Asp Leu Arg Arg Arg Phe Asp Thr Phe 450 Lys Ala Phe Asn Ser Ala Lys Asn Ser Trp Leu Asn Leu Cys Ser Ala 465 Ala Cys Cys Leu Pro Val Ser Thr Ala Tyr His Ile Lys Asn Pro Ile	Ile	Thr	Val	Asn		Asp	Lys	Lys	Asn		Val	Gly	Leu	Leu		Gly
Asp Tyr Pro Pro Leu Ala Thr Asp Leu Arg Arg Arg Phe Asp Thr Phe 450 Phe Asp Thr Ala Tyr Leu Asp Leu Cys Ser Ala 480 Phe Cys Cys Leu Pro Val Ser Thr Ala Tyr His Ile Lys Asp Pro Ile	Cys	Phe	Trp		Ser	Asp	Asp	Glu	_	Cys	Tyr	Val	Gly		Ala	Ser
450 455 460 Lys Ala Phe Asn Ser Ala Lys Asn Ser Trp Leu Asn Leu Cys Ser Ala 465 470 470 Ala Cys Cys Leu Pro Val Ser Thr Ala Tyr His Ile Lys Asn Pro Ile	Phe	Thr	_	Gly	Ser	Ile	His		Ile	Lys	Thr	Leu	_	Val	Tyr	Ser
465 470 475 480 Ala Cys Cys Leu Pro Val Ser Thr Ala Tyr His Ile Lys Asn Pro Ile	Asp	_	Pro	Pro	Leu	Ala		Asp	Leu	Arg	Arg	_	Phe	Asp	Thr	Phe
		Ala	Phe	Asn	Ser		Lys	Asn	Ser	Trp		Asn	Leu	Сув	Ser	
	Ala	Cys	Cys	Leu		Val	Ser	Thr	Ala		His	Ile	Lys	Asn		Ile

Gly Gly Val Phe Phe Thr Asp Ser Pro Glu His Leu Leu Gly Tyr Ser 505 Arg Asp Leu Asp Thr Asp Val Val Ile Asp Lys Leu Lys Ser Ala Lys 520 Thr Ser Ile Asp Ile Glu His Leu Ala Ile Val Pro Thr Thr Arg Val Asp Gly Asn Ser Tyr Tyr Trp Pro Asp Ile Tyr Asn Ser Ile Ile Glu Ala Ala Ile Asn Arg Gly Val Lys Ile Arg Leu Leu Val Gly Asn Trp Asp Lys Asn Asp Val Tyr Ser Met Ala Thr Ala Arg Ser Leu Asp Ala Leu Cys Val Gln Asn Asp Leu Ser Val Lys Val Phe Thr Ile Gln Asn Asn Thr Lys Leu Leu Ile Val Asp Asp Glu Tyr Val His Ile Thr Ser 615 Ala Asn Phe Asp Gly Thr His Tyr Gln Asn His Gly Phe Val Ser Phe 630 635 Asn Ser Ile Asp Lys Gln Leu Val Ser Glu Ala Lys Lys Ile Phe Glu 650 Arg Asp Trp Val Ser Ser His Ser Lys Ser Leu Lys Ile <210> SEQ ID NO 5 <211> LENGTH: 314 <212> TYPE: PRT <213> ORGANISM: Artificial Sequence <220> FEATURE: <223 > OTHER INFORMATION: A56R <400> SEQUENCE: 5 Met Thr Arg Leu Pro Ile Leu Leu Leu Leu Ile Ser Leu Val Tyr Ala Thr Pro Phe Pro Gln Thr Ser Lys Lys Ile Gly Asp Asp Ala Thr Leu Ser Cys Asn Arg Asn Asn Thr Asn Asp Tyr Val Val Met Ser Ala Trp Tyr Lys Glu Pro Asn Ser Ile Ile Leu Leu Ala Ala Lys Ser Asp Val Leu Tyr Phe Asp Asn Tyr Thr Lys Asp Lys Ile Ser Tyr Asp Ser Pro Tyr Asp Asp Leu Val Thr Thr Ile Thr Ile Lys Ser Leu Thr Ala Arg Asp Ala Gly Thr Tyr Val Cys Ala Phe Phe Met Thr Ser Thr Thr Asn Asp Thr Asp Lys Val Asp Tyr Glu Glu Tyr Ser Thr Glu Leu Ile Val Asn Thr Asp Ser Glu Ser Thr Ile Asp Ile Ile Leu Ser Gly Ser Thr 135 His Ser Pro Glu Thr Ser Ser Lys Lys Pro Asp Tyr Ile Asp Asn Ser 150 155 Asn Cys Ser Ser Val Phe Glu Ile Ala Thr Pro Glu Pro Ile Thr Asp 170 Asn Val Glu Asp His Thr Asp Thr Val Thr Tyr Thr Ser Asp Ser Ile 185

Asn Thr Val Ser Ala Ser Ser Gly Glu Ser Thr Thr Asp Glu Thr Pro 200 Glu Pro Ile Thr Asp Lys Glu Asp His Thr Val Thr Asp Thr Val Ser 215 Tyr Thr Thr Val Ser Thr Ser Ser Gly Ile Val Thr Thr Lys Ser Thr Thr Asp Asp Ala Asp Leu Tyr Asp Thr Tyr Asn Asp Asn Asp Thr Val Pro Pro Thr Thr Val Gly Gly Ser Thr Thr Ser Ile Ser Asn Tyr Lys Thr Lys Asp Phe Val Glu Ile Phe Gly Ile Thr Ala Leu Ile Ile Leu Ser Ala Val Ala Ile Phe Cys Ile Thr Tyr Tyr Ile Tyr Asn Lys Arg Ser Arg Lys Tyr Lys Thr Glu Asn Lys Val <210> SEO ID NO 6 <211> LENGTH: 370 <212> TYPE: PRT <213> ORGANISM: Artificial Sequence <220> FEATURE: <223> OTHER INFORMATION: FZD-ECD-A56R Fusion Protein <400> SEOUENCE: 6 Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly Ala His Ser Phe Gly Asp Glu Glu Glu Arg Arg Cys Asp Pro Ile Arg 25 Ile Ser Met Cys Gln Asn Leu Gly Tyr Asn Val Thr Lys Met Pro Asn Leu Val Gly His Glu Leu Gln Thr Asp Ala Glu Leu Gln Leu Thr Thr Phe Thr Pro Leu Ile Gln Tyr Gly Cys Ser Ser Gln Leu Gln Phe Phe Leu Cys Ser Val Tyr Val Pro Met Cys Thr Glu Lys Ile Asn Ile Pro Ile Gly Pro Cys Gly Gly Met Cys Leu Ser Val Lys Arg Arg Cys Glu Pro Val Leu Lys Glu Phe Gly Phe Ala Trp Pro Glu Ser Leu Asn Cys Ser Lys Phe Pro Pro Gln Asn Asp His Asn His Met Cys Met Glu Gly Pro Gly Asp Glu Glu Val Pro Leu Pro His Lys Thr Pro Ile Gln Pro 150 155 Gly Glu Glu Thr Ser Thr Thr Asn Asp Thr Asp Lys Val Asp Tyr Glu 170 Glu Tyr Ser Thr Glu Leu Ile Val Asn Thr Asp Ser Glu Ser Thr Ile 185 Asp Ile Ile Leu Ser Gly Ser Thr His Ser Pro Glu Thr Ser Ser Lys 200 Lys Pro Asp Tyr Ile Asp Asn Ser Asn Cys Ser Ser Val Phe Glu Ile 215 220 Ala Thr Pro Glu Pro Ile Thr Asp Asn Val Glu Asp His Thr Asp Thr 230 235

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Val Thr Tyr Thr Ser Asp Ser Ile Asn Thr Val Ser Ala Ser Ser Gly 250 Glu Ser Thr Thr Asp Glu Thr Pro Glu Pro Ile Thr Asp Lys Glu Asp His Thr Val Thr Asp Thr Val Ser Tyr Thr Thr Val Ser Thr Ser Ser Gly Ile Val Thr Thr Lys Ser Thr Thr Asp Asp Ala Asp Leu Tyr Asp Thr Tyr Asn Asp Asn Asp Thr Val Pro Pro Thr Thr Val Gly Gly Ser Thr Thr Ser Ile Ser Asn Tyr Lys Thr Lys Asp Phe Val Glu Ile Phe Gly Ile Thr Ala Leu Ile Ile Leu Ser Ala Val Ala Ile Phe Cys Ile Thr Tyr Tyr Ile Tyr Asn Lys Arg Ser Arg Lys Tyr Lys Thr Glu Asn Lys Val 370 <210> SEO ID NO 7 <211> LENGTH: 855 <212> TYPE: PRT <213> ORGANISM: Artificial Sequence <220> FEATURE: <223> OTHER INFORMATION: Her2-A56R Fusion Protein <400> SEOUENCE: 7 Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly 10 Ala His Ser Ser Thr Gln Val Cys Thr Gly Thr Asp Met Lys Leu Arg 25 Leu Pro Ala Ser Pro Glu Thr His Leu Asp Met Leu Arg His Leu Tyr Gln Gly Cys Gln Val Val Gln Gly Asn Leu Glu Leu Thr Tyr Leu Pro Thr Asn Ala Ser Leu Ser Phe Leu Gln Asp Ile Gln Glu Val Gln Gly Tyr Val Leu Ile Ala His Asn Gln Val Arg Gln Val Pro Leu Gln Arg Leu Arg Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr Ala Leu Ala Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr Pro Val Thr Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser Leu Thr 135 Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys Asn Asn Gln Leu Ala Leu Thr Leu Ile Asp Thr Asn Arg Ser Arg Ala Cys His Pro 185 Cys Ser Pro Met Cys Lys Gly Ser Arg Cys Trp Gly Glu Ser Ser Glu 200 Asp Cys Gln Ser Leu Thr Arg Thr Val Cys Ala Gly Gly Cys Ala Arg

Cys 225	Lys	Gly	Pro	Leu	Pro 230	Thr	Asp	Cys	Сла	His 235	Glu	Gln	Cys	Ala	Ala 240
Gly	Сув	Thr	Gly	Pro 245	Lys	His	Ser	Asp	Сув 250	Leu	Ala	CAa	Leu	His 255	Phe
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Ser 705	Val	Phe	Glu	Ile	Ala 710	Thr	Pro	Glu	Pro	Ile 715	Thr	Asp	Asn	Val	Glu 720
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Val	Ser 770	Thr	Ser	Ser	Gly	Ile 775	Val	Thr	Thr	Lys	Ser 780	Thr	Thr	Asp	Asp
Ala 785	Asp	Leu	Tyr	Asp	Thr 790	Tyr	Asn	Asp	Asn	Asp 795	Thr	Val	Pro	Pro	Thr 800
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Phe	Val	Glu	Ile 820	Phe	Gly	Ile	Thr	Ala 825	Leu	Ile	Ile	Leu	Ser 830	Ala	Val
Ala	Ile	Phe 835	Cya	Ile	Thr	Tyr	Tyr 840	Ile	Tyr	Asn	Lys	Arg 845	Ser	Arg	Lys
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Ala 2 865	Asp	Leu	Tyr	Asp	Thr 870	Tyr	Asn	Asp	Asn	Asp 875	Thr	Val	Pro	Pro	Thr 880
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Phe Thr Pro Leu	Ile Gln 70	Tyr Gly	Cys Ser	Ser Gln 75	Leu	Gln	Phe	Phe 80
Leu Cys Ser Val	Tyr Val 85	Pro Met	Cys Thr 90	Glu Lys	Ile	Asn	Ile 95	Pro
Ile Gly Pro Cys		Met Cys	Leu Ser 105	Val Lys	Arg	Arg 110	Cys	Glu
Pro Val Leu Lys 115	Glu Phe	Gly Phe 120	Ala Trp	Pro Glu	Ser 125	Leu	Asn	Cya
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Pro Gly Asp Glu 145	Glu Val 150	Pro Leu	Pro His	Lys Thr 155	Pro	Ile	Gln	Pro 160
Gly Glu Glu Cys	His Ser 165	Val Gly	Thr Asn 170		Gln	Tyr	Ile 175	Trp
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Val Ala Leu Thr 210	Ile Met	Gly Val 215	Ile Phe	Leu Ile 220	Ser	Val	Ile	Val
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Tyr 145	Asn	Asp	Asn	Asp	Thr 150	Val	Pro	Pro	Thr	Thr 155	Val	Gly	Gly	Ser	Thr 160
Thr	Ser	Ile	Ser	Asn 165	Tyr	Lys	Thr	Lys	Asp 170	Phe	Val	Glu	Ile	Phe 175	Gly
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Tyr	Tyr	Ile 195	Tyr	Asn	ГÀЗ	Arg	Ser 200	Arg	ГÀЗ	Tyr	ГÀа	Thr 205	Glu	Asn	Lys
Val	Met 210	Thr	Thr	Pro	Arg	Asn 215	Ser	Val	Asn	Gly	Thr 220	Phe	Pro	Ala	Glu
Pro 225	Met	Lys	Gly	Pro	Ile 230	Ala	Met	Gln	Ser	Gly 235	Pro	Lys	Pro	Leu	Phe 240
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-continued

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What is claimed is:

- 1. An isolated polynucleotide comprising:
- (a) a first nucleic acid fragment that encodes an integral membrane protein (IMP) or fragment thereof, wherein the IMP or fragment thereof comprises at least one extra-membrane region, at least one transmembrane domain and at least one intra-membrane region, and wherein a portion of the first nucleic acid fragment encoding at least one intra-membrane region is situated at the 5' or 3' end of the first nucleic acid fragment; and
- (b) a second nucleic acid fragment that encodes a poxvirus EEV-specific protein or functional fragment thereof, wherein the second nucleic acid fragment is fused in frame to a portion of the first nucleic acid fragment that encodes an intra-membrane region of the IMP;
- wherein a poxvirus infected cell comprising the polynucleotide can express an IMP-poxvirus EEV-specific protein fusion protein as part of the outer envelope membrane of an extracellular enveloped virion (EEV).
- 2. The polynucleotide of claim 1, wherein the IMP is a multi-pass membrane protein comprising at least two transmembrane domains.
- 3. The polynucleotide of claim 2, wherein the IMP has an odd number of transmembrane domains, wherein the 5' end of the first nucleic acid fragment encodes an extra-membrane region, wherein the 3' end of the first nucleic acid fragment encodes an intra-membrane region, and wherein the 5' end of the second polynucleotide is fused to the 3' end of the first nucleic acid fragment.
- **4**. The polynucleotide of claim **3**, wherein the IMP comprises a G-protein coupled receptor (GPCR).
- **5**. The polynucleotide of claim **4**, wherein the IMP is the human frizzled-4 protein (FZD4), or a fragment thereof.
- **6**. The polynucleotide of claim **4**, wherein the IMP is the CXC chemokine receptor CXCR4, or a fragment thereof.
- 7. The polynucleotide of claim 2, wherein the IMP has an even number of transmembrane domains, and wherein both the 5' and 3' ends of the first nucleic acid fragment encode

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intra-membrane regions, and wherein the second nucleic acid fragment is fused to 3' end of the first nucleic acid fragment.

- **8**. The polynucleotide of claim **7**, wherein the IMP is human CD20 protein, or a fragment thereof.
- **9**. The polynucleotide of claim **1**, which is operably associated with a poxyirus promoter.
- 10. The IMP-poxvirus EEV-specific protein fusion protein encoded by the polynucleotide of claim 1.
- 11. A poxvirus genome comprising the polynucleotide of claim 1.
- 12. A recombinant poxvirus EEV comprising a heterologous IMP or fragment thereof fused to a poxvirus EEV-specific protein or membrane-associated fragment thereof, wherein the fusion protein is situated in the EEV outer envelope membrane, wherein the IMP or fragment thereof displays on the surface of the EEV in its native conformation.
- 13. A method to select antibodies that bind to a multi-pass membrane protein comprising:
 - (a) attaching the recombinant EEV of claim 12 to a solid support;
 - (b) providing an antibody display library, wherein the library comprises display packages displaying a plurality of antigen binding domains;
 - (c) contacting the display library with the EEV such that display packages displaying antigen binding domains that specifically binds to the IMP expressed on the EEV can bind thereto;
 - (d) removing unbound display packages; and
 - (e) recovering display packages that display an antigen binding domain specific for the IMP expressed on the EEV.
- **14**. The method of claim **13**, wherein the EEV are attached to the solid surface via reaction with tosyl groups attached to the surface.
- 15. The method of claim 13, wherein the EEV are biotinylated and attached to a streptavidin coated solid surface.

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